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Activity-based anorexia: the effects of resistant starch

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**ACTIVITY-BASED ANOREXIA:
THE EFFECTS OF RESISTANT STARCH**

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science**

in

The School of Human Ecology

By

**Holly M. Nguyen
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ABBREVIATIONS

2-DG	2-deoxy-D-glucose
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindole acetic acid
ABA	Activity-based anorexia
ACTH	Adrenocorticotrophic hormone
AIN-93G	American Institute of Nutrition-93 Growing
AL	Ad Libitum
AN	Anorexia nervosa
ARC	Arcuate
AS	Activity Stress
AUC	Area Under Curve
BCAA	Branch-chained amino acids
BGL	Blood glucose level
BMI	Body mass index
BN	Bulimia nervosa
CCK	Cholecystokinin
CHO	Carbohydrate
CRH	Corticotropin-releasing hormone
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
DA	Dopamine
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders (4 th ed., text revision)

E	Epinephrine
EDNOS	Eating disorder not otherwise specified
EURESTA	European Concerted Action of Resistant Starch
GAL	Galanin
GI	Glycemic index
GIP	Glucose-dependent insulintropic polypeptide
GLP-1	Glucagon-like peptide-1
IBW	Ideal body weight
IGT	Impaired glucose tolerance
IP	Intraperitoneal
LNAA	Large Neutral Amino Acids
NE	Norepinephrine
NPY	Neuropeptide Y
NR	Non-Running
PCPA	Parachlorophenylalanine
POMC	Proopiomelanocortin
PRT	Physical readiness test
PVN	Paraventricular
R	Running
RF	Restricted-feeding
RS (rs)	Resistant starch
SCFA	Short-chained fatty acids
trp	tryptophan

DEFINITIONS

<i>Activity-based anorexia (ABA)</i>	a condition in which subjects maintained on a food restriction schedule and given free access to running wheels display hypophagia, rapid body weight loss, and increased running wheel activity
<i>Anorexia</i>	a loss of appetite especially when prolonged
<i>Anorexia nervosa (AN)</i>	a serious eating disorder that is characterized by a pathological fear of weight gain leading to faulty eating patterns, malnutrition, and usually excessive weight loss
<i>Activity-stress ulcer</i>	a symptomatic model of anorexia nervosa and obsessive-compulsive disorder as well as peptic ulcer; models similar to activity-based anorexia
<i>Bulimia nervosa (BN)</i>	an eating disorder in which a person regularly binges and then uses laxatives, induces vomiting, fasts, or uses extreme exercise in order to prevent weight gain
<i>Cachexia</i>	general physical wasting and malnutrition usually associated with chronic disease
<i>Eating disorders not otherwise specified (EDNOS)</i>	an eating disorder in which the patient meets some but not all of the criteria of another eating disorder
<i>Obesity</i>	being 20% above the IBW or a BMI of ≥ 30
<i>Overweight</i>	being 10-20% above the ideal body weight (IBW) or a body mass index (BMI) of 25-29
<i>Glycemic index</i>	a score to measure the effect of individual foods on blood glucose levels
<i>Resistant starch (RS)</i>	a type of starch that is resistant to the effects of digestive enzymes and is not digested in the small intestine; when consumed, resistant starch functions much like fiber in the human diet

ABSTRACT

Anorexia nervosa is the third most common illness among adolescent females. Approximately one half the cases of anorexia nervosa have been suggested to be activity-induced. Various animal studies have been used to study human anorexia, particularly the activity-based anorexia model (ABA). The ABA paradigm consists of diet restriction and liberal access to activity, which ultimately results in a rapid decrease in both body weight and food intake paradoxical to the significant increase in activity. Because resistant starch (RS) has been shown to initiate a lower rise and a steady level of post-prandial blood glucose, it was hypothesized that a diet containing RS would reduce the severity of the anorexia associated with the ABA model. In this study, 56 five-wk old male Sprague-Dawley rats were assigned to 8 groups. Animals were fed a control diet (C-diet) or a RS-diet, on an ad libitum or a restricted-feeding (one 90min meal per day) schedule, and allowed 22-hr of access or no access to activity wheels. The study ended when a majority of the ABA rats reached $\leq 75\%$ of their pre-experimental body weights. Within 4 days of the experiment, ABA rats on the RS-diet lost an average 66g of body weight compared to an average loss of 31g in the C-diet ($p < 0.01$). ABA rats on the RS-diet ran 31% more (NS), despite consuming 30% fewer calories per kg body weight, than those on the C-diet ($p < 0.01$). ABA rats fed the RS-diet had 3.97 times higher levels of plasma norepinephrine (NE) compared to their associated controls ($p < 0.0001$); ABA rats fed the C-diet had only 1.4 times the NE level of their corresponding controls (NS). All RS-fed rats had an average of 17-50% less fat pad (brown, perirenal, epididymal, & retroperitoneal) weights compared to C-fed rats ($p < 0.02$). Resistant starch exacerbates rather than mitigates the responses to the ABA paradigm.

CHAPTER 1

INTRODUCTION

In the U.S., the high availability of various foods, the sedentary lifestyles, and society's standards for the ideal image in men (Leit, Gray, & Pope, 2002) and women (Groesz, Levine, & Murnen, 2002) have lead to a wide range of nutritional complications. For example, the public encountered and continues to struggle with the problem of obesity. More than sixty percent of the U.S. population is either overweight or obese (Must et al., 1999), and the prevalence of children with obesity is increasing (Birch & Fisher, 1998). Overweight is defined as being 10-20% above the ideal body weight (IBW) (Forsythe, 1998) or a body mass index (BMI) of 25-29 (Laquatra, 2000). Obesity is defined as being 20% above the IBW (Forsythe, 1998) or a BMI of ≥ 30 (Laquatra, 2000). The medical consequences of obesity include, but are not limited to, increased risks of cardiovascular diseases, joint diseases, gall bladder diseases, cancer, diabetes, hypertension, lipid disorders, and gout (Forsythe, 1998; Laquatra, 2000; Must et al., 1999).

On the other hand, there are people who are struggling with severe underweight and eating disorders, both of which can eventually lead to a complex state of malnutrition or death if left untreated. Eating disorders are considered a physiological or a psychological problem, depending on the disorder. One of the most common types of eating disorders is anorexia nervosa. Anorexia nervosa (AN) is an eating disorder characterized by the failure to maintain a body weight within 15% of one's IBW, distorted food beliefs or food aversions, intense fear and refusal of gaining weight, excessive weight loss, amenorrhea in females, and sometimes obsessive activity.

Individuals with AN have a distorted body image, that is, they perceive themselves as being overweight despite their emaciated status, and they are often in denial of their condition (Forsythe, 1998; Schebendach & Reichert-Anderson, 2000). However, it is well worth stating that the literal meaning of anorexia is the loss of appetite, especially when prolonged.

Anorexia nervosa can lead to many medical outcomes that include a weakened immune system, anemia, delayed gastric emptying, organ damages, irregular heartbeats, changes in the central nervous system, low body temperatures, low blood pressures, pubertal delay in children, and the disruption of menstrual cycles in females (Schebendach & Reichert-Anderson, 2000). It has been estimated that approximately 6% of anorexics die from anorexia nervosa (Forsythe, 1998). If AN is detected and treated early in the disorder, there can be a decline in the mortality rate by 10%, and up to 80% of the involved individuals may either fully or partially recover (Becker, Grinspoon, Klibanski, & Herzog, 1999).

Past research suggested that one in every 800-1000 females are associated with anorexia nervosa, but it is now estimated that one out of every hundred young females have the disorder (Forsythe, 1992). The most common period of onset seems to be during adolescence (Schebendach & Reichert-Anderson, 2000). Girls between the ages of 9-14 have reported a desire to be thinner and have attempted dieting (Halvarsson, Lunner, Westerberg, Anteson, & Sjoden, 2002). More attention is given to females because the majority of the reported cases are primarily females; however, the rate of incidence in males is increasing (Forsythe, 1998). Males account for approximately 5-10% of all eating disorders (Carlat, Camargo, & Herzog, 1997), and it is suggested that

incidences for males are lower than that for females because males tend to be less dissatisfied about their weight (Sisson, Franco, Carlin, & Mitchell, 1997). Other researchers, however, have reported findings that males were more disturbed about their weight than females (Sorbara & Geliebter, 2002). Nevertheless, whether males or females are more displeased about their weights, it has been strongly indicated that the media plays a large role in rendering the negative perception about body size, shape and weight (Groesz et al., 2002; Leit et al., 2002).

A common characteristic of individuals with anorexia nervosa is increased physical activity. Furthermore, it has been suggested that this increase in activity is one of the first signs to appear abnormal and one of the last few to normalize (Kron, Katz, Gorzynski, & Weiner, 1978). Therefore, the proposed study is designed to model the anorexia found in highly active individuals. Because the weight loss of anorexia can be attributed in part to the combination of low energy intakes and high-energy expenditures, there must be a way to ameliorate the weight loss effect—if not entirely preventing it from occurring. The best probable alternative may be to prolong the progression to anorexia, rather than solely attempting to reverse it.

In the proposed study, young male rats are subjected to the standard activity-based anorexia (ABA) model that consisted of a simultaneous exposure to restricted feeding schedules and liberal access to running wheels. Activity-based anorexia (ABA) can be defined as the reduction of food intake and a progressive loss of body weight that is paradoxical to an increase in wheel running. In the intended study, it was predicted that the rats would continue to run while their food intake is decreased. Because there is no compensation for the energy expended on the running wheels, the rats will continue to

lose weight. The result of this procedure, if allowed to continue for several days, can lead to severe loss of body weight and eventual death if the rats are not removed from the experiment. ABA has clinical significance because some patients with anorexia nervosa often use intense exercising and diet restriction as a means of losing weight and maintaining the weight loss (C. Davis, Kennedy, Ravelski, & Dionne, 1994; Kron et al., 1978).

Objective and Hypothesis

The objective of this study is to determine whether the outcomes of the activity-based anorexia (ABA) paradigm can be attenuated if the diet contained resistant starch. It is hypothesized that if the animals are fed a diet containing resistant starch while exposed to the standard ABA procedure, the effects of the ABA model will be ameliorated.

Rationale and Justification

A high glycemic diet produces transient post-prandial hyper- and hypo-glycemia. Conversely, because animals fed a resistant starch, which has a low glycemic index, slowly digest and absorb glucose, they are able to maintain euglycemia for a longer period of time when compared to those fed a high glycemic index diet (Raben, Kiens, & Richter, 1994). Because euglycemia is sustained over a longer time frame, there should be less, if any, stimulation of catabolic hormone release, and thus, energy stores will not be catabolized. Higher body stores of energy (e.g., fat, protein, glycogen) are suggested to attenuate wheel running associated with ABA (Fish & Lewis, 1996); therefore, it is possible that a resistant starch diet may attenuate the outcomes of ABA.

Assumptions

1. The activity-based anorexia paradigm is an acceptable model of human anorexia.
2. The blood and tissue collections from the animals are representative of humans.
3. The animals are free from any diseases or disorders that may affect the results.
4. The design of the run-test(s) will allow appropriate determination for the assignment of animals to specified groups.

Limitations

1. Psychological issues, which are evident in human anorexia nervosa, cannot be evaluated in rats, thus the model lacks psychological representation of this disorder.
2. Possible gender differences were not identified because experiment(s) did not simultaneously consist of both male and female rats due to limited laboratory space and equipment.
3. The number of animals used may not be sufficient to generate a reliable conclusion.
4. The intensity, frequency, and duration were not measured due to lack of equipment and programs.

CHAPTER 2

REVIEW OF LITERATURE

Societal influences have put a great deal of pressure on its people. Overemphasis on health, fitness, beauty, and achievements has caused many to be at harm. For example, males and females struggle to be thin and fit in order to adhere to the standard of the ideal man or woman (Silverstein & Perdup, 1998). Athletes in competitive sports strive to be at certain body weights in order to compete at their maximum potential (Stoutjesdyk & Jevne, 1993). Military soldiers are expected to put great effort in reaching or maintaining body weight within strict standards. A report by McNulty (1996) indicated that many people in the U.S. Army, Navy, Marines, and Air Force were discharged or failed to get a promotion because they were overweight (McNulty, 2001). In a study by Rose et al. (1993), Army soldiers indicated that appearance, health concerns, and upcoming weigh-ins were the primary reasons for attempting weight loss (McNulty, 2001). However, there is danger when dieting individuals develop eating disorders. They may become emotionally obsessive about their appearance, which may result in abnormal eating patterns (i.e., bingeing, purging, starving) or abnormal exercising routines to a point of severe malnourishment and physiological and psychological complications (Becker et al., 1999; Rock, 1999).

Eating disorders affect people of all ages, especially adolescent females, for whom it is the third most common chronic illness (Kreipe & Birndorf, 2000). Over five million Americans suffer from some form of eating disorder (Adolescent Medicine Committee & Canadian Paediatric Society, 1998). Research shows that more females are reported to have eating disorders than are males, who make up only 5-10% of the

population with eating disorders (Carlat et al., 1997). In addition, there is a higher prevalence among explicit groups in which diet restriction and regulation of body weight is of great importance (e.g., ballerinas, dancers, marathon runners, or models) (Carlat et al., 1997; Engstrom et al., 1999). Because eating disorders are more than merely medical complications, they are usually treated with the help of an interdisciplinary team (e.g., medical professionals, nurses, dietitians, and psychiatrists), family, relatives, and close friends (Adolescent Medicine Committee & Canadian Paediatric Society, 1998).

Examples of eating disorders include, but are not exclusive to, anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified (EDNOS). EDNOS is a less severe form of AN and BN that is estimated to occur in approximately 1-2% of the population and makes up 50% of all eating disorders. An individual with an eating disorder is classified as having EDNOS if he or she has not met all the criteria of any specific eating disorder. For example, in the fourth edition of *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV-TR), a female is diagnosed with having EDNOS if she has met all criteria for AN but continues to have regular menses. EDNOS, however, can develop into AN or BN if left undiagnosed and untreated (Spear & Stollefson, 2001).

Bulimia nervosa is an eating disorder characterized by repeated episodes of bingeing with subsequent purging and feelings of guilt. Unlike anorexia nervosa, individuals with BN are usually at normal or above normal weights. According to the DSM-IV-TR, in order to be diagnosed with BN, an individual (1) must have “recurrent episodes of binge eating” and a “recurrent inappropriate compensatory behavior,” in an attempt to prevent weight gain, for at least twice a week for a period of three months; (2)

must evaluate his or her self by means of body shape and weight; and (3) must *not* be diagnosed with anorexia nervosa. BN occurs in 2-5% of the population (Spear & Stollefson, 2001).

Anorexia nervosa is a psychiatric disorder in which individuals have distorted body images, even if underweight, and have distorted food beliefs. The individuals are usually $\leq 85\%$ of their ideal body weight (IBW) or have a body-mass index (BMI) of ≤ 17.5 (Becker et al., 1999). The DSM-IV-TR states that to be diagnosed as having AN, one must (1) refuse to maintain body weight at or above standard weight for one's age and height; (2) have an intense worry of weight gain or fat gain even if one is underweight; (3) have a distorted body image; and (4) have amenorrhea if she is post-menarcheal (Spear & Stollefson, 2001). AN is the only psychiatric disorder that requires a biological abnormality (i.e., amenorrhea) for diagnosis in females (C. Davis, 1997). Death from AN is usually due to starvation, suicide, or electrolyte imbalance (McNulty, 2001).

Many health complications result from eating disorders. For example, in AN, symptoms can range from brittle hair and nails, dry skin, severe constipation, hypoglycemia, and hypotension to bradycardia, delayed gastric emptying, osteoporosis, and other cardiovascular complications (e.g., reduced heart mass) (Bachrach, Guido, Katzman, Litt, & Marcus, 1990; Becker et al., 1999; Cooke & Chambers, 1995; Fisher et al., 1995; Kreipe & Birndorf, 2000; Rock, 1999; Swenne, 2000; Webb, Kiess, & Chan-Yan, 1986). In addition, BN symptoms include gastrointestinal problems, esophageal reflux disease, muscle weakness, fatigue, cardiac arrhythmias, dehydration, electrolyte imbalances, and dental erosion (Mitchell, Hatsukami, Eckert, & Pyle, 1985; Rock, 1999).

Furthermore, in any of the eating disorders the medical complications may be deemed irreversible (i.e., retarded growth or hindered puberty) if the individual is not diagnosed and treated (Abrams et al., 1993; Fisher et al., 1995).

Human Studies

In 1997, 1.1% of the active duty U.S. female Navy nurses who participated in a study were diagnosed with anorexia nervosa, which was comparable to the incidence of AN in the U.S. national population (McNulty, 1997a). However, researchers found that the incidence of BN was six times greater for the Navy nurses than that of the U.S. population. Another study in 1997 showed that there was a 2.5% and 6.8% incidence of AN and BN, respectively, in a study of 4,800 Navy men (McNulty, 1997b).

Edholm and associates reported that cadets ingest less food on days of military drilling than they do on days of lower activity (Edholm, Fletcher, Widdowson, & McCance, 1955). In a correlational study of 1,278 anonymous females in the armed services (U.S. Army, Navy, Air Force, and Marines in Okinawa, Japan), surveys showed that body fat averaged 19.5% for the total sample and amenorrhea was significantly higher among members of the Marines—this was found to reflect the youngest population. The Marines also reported the highest use of diet pills and laxatives and the highest rate of vomiting. Participants indicated increased episodes of bingeing or purging during the biannual Physical Readiness Test (PRT). The surveys showed that the most frequently used method for attaining one's appropriate weight was fasting, followed by bingeing and the use of diet pills (McNulty, 2001).

Reports suggest that much of human anorexia may be activity-induced (Crisp, Hsu, Harding, & Hartshorn, 1980; C. Davis et al., 1994; King, Burley, & Blundell, 1994).

Exercise has been known to reduce weight in some individuals and possibly the amount of food consumption per day if all other factors (e.g., age, sex, diet, duration and intensity of exercise) are taken into account (Durrant, Royston, & Wloch, 1982; Pi-Sunyer & Woo, 1985; Thompson, Wolfe, & Eikelboom, 1988). Nonetheless, the connection between activity and food consumption primarily contributes to the balance of energy (King et al., 1994). The effects of exercise on energy intake in normal-weight young people (10 men and 10 women) were examined. Participants were given a diet individualized to their needs and were randomly assigned to either an exercising group (1 hour/day of treadmill at ~68% of VO_2max for five days) or to a sedentary group (no treadmill exercise). Although there was a significant increase in caloric intake by exercising men and no significant increase for exercising women, all the active participants were in negative energy balance during the exercise period (Staten, 1991).

In another study, the effect of intensity and duration of exercise on energy consumption was studied in twenty-three healthy male participants. In part one of the study, the participants were assigned to a control group, a low-intensity exercising group, or a high-intensity exercising group. In part two of the study, participants were assigned to a short-duration exercising group, a high-duration exercising group, or a control group. In part one, there was no reduction of hunger in those in the low-intensity group at any time. Conversely, there was a short-lived suppression of hunger during exercise and 15 minutes immediately following exercise for those in the high-intensity group. There was no effect on total caloric consumption among any groups. In part two, there was a significant decrease in hunger during both the short- and long-duration exercise periods.

In addition, hunger suppression continued after exercising with the greatest suppression for those in the long-duration exercise treatment (King et al., 1994).

Animal Models

Animal models of anorexia have been used for nearly 50 years (J. F. Hall & Hanford, 1954). Many animal studies used rats as a model for human anorexia, but the animal paradigm has also been performed with mice (Epling, Pierce, & Stefan, 1983), hamsters, gerbils, and guinea pigs (Vincent & Pare, 1976). It was Epling and co-authors who coined the term “activity-based anorexia” and proposed a link between anorexia in animals to anorexia nervosa in humans (Epling et al., 1983). However, activity-based anorexia may also be referred to as “self-starvation” (Routtenberg & Kuznesof, 1967; Spatz & Jones, 1971) or “activity-stress” procedures (Pare & Houser, 1973). The former was the original name given to this paradoxical effect and the latter is usually used in the context of studies focusing on stomach ulcerations. Therefore, because activity-based anorexia is a term widely used, it will be used throughout the present literature review to refer to the negative effects that result from severe food restriction and excessive activity.

Activity-based anorexia (ABA) has been typically characterized by a reduction in food intake and body weight paradoxically accompanied by a progressive increased activity level (Dwyer & Boakes, 1997). A standard activity-based anorexia (ABA) animal model includes simultaneous introduction of diet restriction (one meal for 60-90 minutes per day) and access to running wheels (21-22.5 hours of access per day) to the animals in the experimental condition. For example, in a study that restricted the mealtime to 60 minutes per day and allowed access to running wheels for the remainder of the day, the animals continued to increase their activity, reduce their food intake, and

lose weight on succeeding days. Over a short period, the animals died because the amount of calories consumed failed to compensate for the energy expended from the running (J. F. Hall & Hanford, 1954; Routtenberg & Kuznesof, 1967). A typical rat would display an initial gradual rise in activity levels and then show a dramatic increase if the experiment was allowed to continue. Moreover, as activity levels increased (i.e., more than 5,000 revolutions per day), the rats' food intake and body weight declined considerably (Beneke, Schulte, & vander Tuig, 1995; Epling & Pierce, 1984).

In a study by Tokuyama and lab, rats showed an initial transient reduction in food intake and a rapid increase in running for the first 5-10 days, followed by an increase in food consumption that surpassed the amount consumed by the controls (Tokuyama, Saito, & Okuda, 1982). Studies have shown that experimental rats can run more than 1 km per day (Boakes, 1997) and eat less than 1 gram per day (Routtenberg & Kuznesof, 1967). Thus, as Epling and co-authors state: "animal research on self-starvation suggests that the combined effects of these variables are multiplicative rather than additive (p. 41)" (Epling et al., 1983). Much research has been performed in an attempt to determine the reason behind this phenomenon. Some researchers suggest that the animals drastically increase their running in order to raise their body temperatures, because animals that fail to adapt to the restricted feeding schedules were found to have low body temperatures (Lambert, 1993). Nevertheless, there are many controversial issues such as whether or not the introduction of both stress factors (i.e., novelty to wheels and deprivation of food) must be presented simultaneously (Ness, Marshall, & Aravich, 1995), whether females or males are more vulnerable to this procedure (Boakes & Dwyer, 1997; Doerries, Stanley, & Aravich, 1991), or whether decreased food intake causes increased running or vice

versa (Boakes & Dwyer, 1997; Epling & Pierce, 1988; Lett, Grant, Smith, & Koh, 2001). Because the majority of the experiments prevented running during the feeding period, the effects of ABA cannot be attributed to the competition between food and activity.

Routtenberg and Kuznesof defined anorexia in the rat as the consumption of less than 1 gram of food (Routtenberg & Kuznesof, 1967). However, because the older definition was too rigorous, animals are now classified as being “anorexic” if their body weight has declined to 70% of their initial body weight, which is the weight loss criterion used more in the context of activity-stress ulcers (Doerries et al., 1991; Epling & Pierce, 1984). Animals are considered to have reached the “removal” or “starvation” criterion if they have reached 75% of their pre-experimental body weight on two consecutive days (Beneke et al., 1995; Boakes, Mills, & Single, 1999). Animals are said to have met the “recovery” or “survival” criterion when their weight on day 4 is at or above the weight on day 1 in any consecutive 4-day period (Beneke et al., 1995; Epling & Pierce, 1984).

Pre-existing characteristics such as pre-experimental body weight, age, and gender seem to play a role in achieving the ABA results. Boakes and Dwyer found that male rats with low initial body weights (180g on average) lost weight faster than those with higher initial body weights (205 on average) although all rats were at the same age (Boakes & Dwyer, 1997). Routtenberg and Kuznesof indicated that rats weighing approximately 270g at the start of an experiment are highly vulnerable to the ABA effect (Routtenberg & Kuznesof, 1967). Rieg and associates suggested that an animal with an initial body weight of more than 350g is less likely to reach a 25% weight loss (Rieg, Maestrello, & Aravich, 1994). Woods and Routtenberg found that age was a very important factor in the vulnerability of male rats to ABA. They discovered that younger

male rats (less than 10 weeks old) lost weight faster than did the older rats (over 10 weeks old). Because the growth rate is quicker for males and especially rapid over the first 70 days of a rat's life, maturation is a confounding variable that should be considered (Woods & Routtenberg, 1971).

Some studies have evaluated gender as another variable in ABA experiments; the results, however, are lacking consistency. A study by Boakes and co-workers included two female groups and one male group in an attempt to match age, weight, and gender factors. One female group (FY; 52 days old; 160g) was age-matched to the male group (MY; 52 days old; 230g). A second female group (FO; 136 days old; 237g) was weight-matched (mean value of ~230-237g) to the MY group. Results of the study demonstrated that both age and gender, rather than weight, are strong factors in the standard ABA procedure; however, age seems to be a more critical aspect in this study. The FY and MY groups reached the "removal" criteria in an average of 7.0 and 7.4 days, respectively. However, it took the FO group an average of 10.83 days to reach the removal criteria (Boakes et al., 1999). These findings are consistent with another lab that found that female rats are more vulnerable to the effects of diet restriction and excessive activity than are male rats (Pare, Vincent, Isom, & Reeves, 1978).

In a different study, researchers attempted to match the weights and age for both male and female rats. In contrast to the findings above, these researchers found that females ran and ate more than the males, and the male rats lost weight faster than the female rats, even though the male rats weighed a mean value of 10g more than the female rats weighed (Doerries et al., 1991). Consistent with this study in some aspects, an even earlier study evaluated the effects of running and food restriction on male and female

rats. They found no differences in food consumption and weight changes between sedentary males and sedentary females. However, they did find that active female rats recovered from the reduced food intake faster than did the active males. Even though, the exercising females ran consistently more (a plateau on day 10 of 11,000 revolutions per day) than did the exercising males (a plateau on day 10 of 4,000 revolutions per day) throughout the 50-day experiment (Tokuyama et al., 1982).

Roy and Wade found that estrogen is an activity-promoting hormone in both male and female rats. Their finding supported the argument that females acquire some characteristics that promote higher activity than do males (E. J. Roy & Wade, 1975). In another study that attempted to determine whether activity levels and survival rates were affected by rats (males and females) subjected to gonadectomy and introduced to the activity-stress (AS) paradigm, researchers found that the castrated animals showed a reduction in activity but there were no sex differences in activity levels. They also found that the males survived longer than the females (6.0-6.3 versus 4.1-4.3 days), and the castrated rats died earlier than the sham-operated rats. Because the animals in the study were castrated and matched according to age, sex, and weight, it was proposed that when these three factors are controlled, the difference in vulnerability between males and females is diminished (Lambert & Kinsley, 1993).

Activity-based Anorexia (ABA) Variations

Prior exposure to deprivation stress or novelty stress has been found to affect the results of the activity-based anorexia paradigm. However, questions remain as to which determinant, for example, variations in activity patterns, meal frequencies, or housing conditions, play a more critical role. Routtenberg isolated the variables relating to ABA

in an attempt to further study the paradoxical effect of the ABA paradigm. He defined “deprivation stress” as the stress resulting from the introduction of food restriction and “novelty stress” as the stress related to exposure to a new environment (running wheels). It was concluded that deprivation stress was essential for the “starvation” effect and novelty stress only reinforced these effects (Routtenberg, 1968).

Kanarek and Collier proposed that the introduction of activity might interact with a satiety mechanism that causes the termination of eating and that the initiation of ABA is caused by a failure to adapt to a restricted feeding schedule (Kanarek & Collier, 1979). Since then, much research has been done to pre-expose and adapt the laboratory animals to one of the two stress factors before administering the second stress factor. In one study, researchers have found that pre-exposure to wheel running increases the rate at which weight loss occurs when a diet restriction is later imposed (Boakes & Dwyer, 1997). In a different study, running alone dramatically reduced body weight to the extent that the experiment was terminated before food restriction was introduced (Verna Rose Burden, 1992).

Unlike pre-adaptation to running, researchers tested the effects of pre-adaptation to the restricted feeding schedule and determined the minimum feeding period required for severe weight loss and ulcer development. The rats were divided into twelve treatment groups. Six groups were pre-adapted to either a 1-hour or a 2-hour feeding schedule and the other six groups remained on ad libitum feeding. All animals were further divided into groups receiving 9, 18, or 24 days of acclimation to the feeding schedules. When access to running wheels was introduced, the pre-adapted animals remained on the same feeding schedules while the freely fed animals received the same

restriction as those of the pre-adapted group. Results showed that with exposure to the wheels, pre-adapted rats ate more than the freely fed rats. In addition, the animals allowed the longest acclimation period ate and weighed more than those allowed shorter acclimation periods. The non-adapted animals had a larger decline in body weight when allowed access to running wheels (Pare, Vincent, & Natelson, 1985).

Consistent with these findings other researchers also found that pre-adapted rats lost less weight and ate more than the non-adapted rat, which led to the conclusion that prior adaptation to the feeding schedule can reduce the severity of ABA outcomes (V. R. Burden, White, Dean, & Martin, 1993; Dwyer & Boakes, 1997). Contrastingly, Lett and co-workers found that pre-adaptation to diet restriction did *not* eliminate ABA in rats. The experimental animals that were adapted to the feeding schedule still ate less, weighed less, and ran more than those of the control group (Lett et al., 2001). Nevertheless, researchers concluded that if the paradigm was not as stringent as the typical ABA procedure (i.e., feeding period of up to 2.5 hours during adaptation period and allowing only 2 hours of wheel access prior to feeding rather than 90 min meal period and 22.5 hr of access to wheel-running), rats who are pre-adapted to the restricted feeding schedule show faster recovery than those who are non-adapted (Boakes & Dwyer, 1997).

The duration of the meal period also appears to take part in attaining anorexia. For example, experimental rats were fed for 30, 45, or 60 minutes per day and were restricted from activity one hour before feeding time. The control rats were restricted on the same feeding schedule as described earlier but did not have access to activity wheels. The results showed that all the animals in the experimental groups “starved” (starvation criterion was defined as eating less than 1 gram during the feeding period) except for one

rat from the 60-minute group, and all the animals in the 30-minute control groups “starved” compared to one animal in the 60-minute control group (Routtenberg & Kuznesof, 1967). Comparably, researchers found that ABA was more likely to be found when the rats were fed 60 minutes per day rather than 120 minutes per day, and it was more likely to occur when the rats were given a single feeding rather than multiple feedings (Kanarek & Collier, 1979; Pare et al., 1985). For example, when there was a variation in the frequency of the meals throughout the day, i.e., four 15-min, two 30-min and one 60-min meal, animals fed the one 60-min meal demonstrated anorexia characteristics as opposed to those fed four 15-min meals. It was suggested that ABA resulted from the failure to overcome satiety signals rather than solely from an activity-induced food intake suppression (Kanarek & Collier, 1983).

It has long been determined that rats are nocturnal eaters and there are two peak eating periods—one at the onset of the dark cycle and the other at the end of the dark cycle (Dwyer & Boakes, 1997; Kersten, Strubbe, & Spiteri, 1980). Thus, to raise the intricacy of adapting to the restricted feeding schedule, most experiments schedule the feeding times during the light cycle. In an attempt to further study this phenomenon, researchers tested whether the time of day of feeding has an effect on adaptation to a restricted feeding schedule. The results showed that animals adapted to the feeding schedule faster when fed during the dark cycle than when fed during the light cycle (Dwyer & Boakes, 1997). Furthermore, Burden and lab, which wanted to control for circadian rhythms in order to measure metabolites in the hypothalamic-pituitary-adrenal axis, demonstrated that animals fed during the dark cycle could still reach the state of activity-based anorexia (Verna Rose Burden, 1992).

It is suggested that variable access to running wheels may have an effect on the typical ABA outcome. For example, Epling and Pierce allowed varying amounts of time of wheel access (2, 6, 12, 18, or 22 hours per day) for the experimental rats that were restricted to a daily 90-minute feeding schedule. They found that rats that had more than 12 hours of access to running wheels indicated evidence of severe ABA (Epling & Pierce, 1984). In another example, rats were allowed 30 minutes of feeding time per day and restricted from activity for either up to 2 hours before or up to 2 hours after feeding period. All of the experimental animals died within a mean number of 13.5 days. In a further experiment, these researchers allowed the rats a 60-minute feeding period in which wheel running was restricted for up to 3 hours before the meal. The results showed that three out of five rats starved (Routtenberg & Kuznesof, 1967).

Likewise, Dwyer and Boakes found that ABA was indicated in rats that were allowed activity for 4 hours prior to the feeding period rather than those allowed activity for 18.5 hours after the feeding period. The rats demonstrated corresponding running intensity except those who were allowed the 4-hour running time before the eating period reduced their food intake. Therefore, it may seem essential to allow activity before feeding to obtain the ABA effect (Dwyer & Boakes, 1997). Epling and Pierce suggested that running alone could cause a decrease in food intake and body weight, regardless of diet restriction. They concluded that a severe reduction in food intake would only occur if activity “exponentially” increases; however, if activity remains stable or slightly increases, the amount of food consumed will eventually counterbalance the amount of energy expended (Epling & Pierce, 1988). As mentioned previously, a study by Burden

demonstrated significant weight loss from running such that the experiment ended before the introduction to food restriction (Verna Rose Burden, 1992).

Housing conditions can also influence the outcomes of the ABA paradigm. The effects of isolation and communal housing on self-starvation were studied. Rats were restricted to 60 minutes of feeding and allowed 22.5 hours of activity before the meal period. Half the animals in the communally housed groups “self-starved” (eating ≤ 1 gram of food per day), but none of those in isolation did (Spatz & Jones, 1971). Animals moved from individual housing to paired-housing showed a decrease in food intake as well (O'Connor & Eikelboom, 2000). Conversely, other researchers found that individually housed animals are slower to adapt to the feeding schedule than the animals in the group-housing condition. Additionally, rats that were previously housed communally and subsequently placed in isolation showed a higher decrease in body weight and a slower recovery rate than those that remained communally-housed (Boakes & Dwyer, 1997; Boakes et al., 1999).

Pathology

As mentioned earlier, activity-based anorexia can result in gastric ulcers. In this situation, the procedure is referred to as the activity-stress (AS) ulcer paradigm (Manning, Wall, Montgomery, Simmons, & Sessions, 1978; Pare & Houser, 1973). The AS paradigm involves restricting meal times to 30-60 minutes per day and allowing access to running wheels for the remainder of the day (Pare, 1975). Similar to ABA, the animals will display high levels of activity and reduced food intake; however, they also develop glandular lesions that penetrate the muscularis mucosa of the gastric lining. More than half the animals constrained to this procedure die within 3-12 days. Thus, this

paradigm has been suggested as a model for peptic ulcers in humans (Lambert, 1993; Manning et al., 1978). Researchers studied the incidence of ulcerogenesis via other methods such as using shock (Murison & Olafsen, 1991) or restraint techniques (Brodie & Hanson, 1960; Politch & Herrenkohl, 1984); however, as acknowledged by Lambert, using diet restriction and allowing access to voluntary activity is the most realistic method if the results are to be compared with humans (Lambert, 1993).

In a study previously mentioned, rats given a shorter acclimation period with access to food *ad libitum* produced the most ulcers when exposed to running wheels and a 1-hour restricted-feeding schedule compared to those allowed pre-adaptation to the 2-hour restricted-feeding schedule (Pare et al., 1985). Consistent with these results, a group of rats pre-adapted to a 1-hour feeding schedule ran faster than the group of rats deprived of the pre-adaptation period, yet the rats in the former group did not develop ulcers, while signs of ulcerations were seen in the latter group (Morrow & Garrick, 1993). Tsuda and co-authors suggested that gastric disease would not occur if rats were given food *ad libitum* while exposed to the running wheels. The incidence of gastric disease or ulcerogenesis would also diminish if rats were given access to running wheels with two 30-minute feeding sessions rather than one 60-minute feeding session. (Tsuda, Tanaka, Iimori, Ida, & Nagasaki, 1981).

In a study referenced earlier, when male and female rats were castrated and exposed to the AS paradigm, there was a significant difference in ulceration, with the castrated male rats having 5.9% of the corpus ulcerated compared to 3.4% ulceration in the castrated female rats (Lambert & Kinsley, 1993). In other studies that examined the effects of gender, significant differences in weight loss between male and female rats

were found, although researchers did not find any sex differences in ulcer sensitivity when the animals were exposed to the AS paradigm (Doerries et al., 1991; Pare et al., 1978).

Other tissues are also affected by a severe diet restriction and intensive exercise, including the thymus, spleen, and adrenal glands. Hara and lab examined these organs as well as the lungs and stomachs after the experimental rats were exposed to either a 60-minute daytime feeding schedule or to a 60-minute nighttime feeding schedule in combination with access to running wheels throughout the experiment. Rats that were restricted to 60 minutes of feeding per day with no access to running wheels, and rats that had access to activity wheels while on ad libitum feeding, revealed no signs of stomach ulcers. However, there were signs of ulcers for those rats that were restricted to food (night and daytime meals) and allowed access to running wheels. Whereas the rats that were fed during the nighttime had a higher occurrence of ulcers compared to those who were fed during the daytime, the latter group died earlier (day 6) than the former group (day 10). In addition, the thymus and spleen weights of the two groups were lower than normal, and the adrenal weights increased above normal. The organ weights of the rats that were on a food-restricted diet with no access to activity wheels also changed considerably, although the changes were only minor compared to the rats that were food-restricted and allowed access to activity. There were reports of bleeding and inflammation of the thymuses as well as bleeding and congestion of the lungs for most of the experimental animals (Hara, Manabe, & Ogawa, 1981).

In another experiment, the incidence of gastric ulcers and the changes in thymus and adrenal glands in male rats were examined. Experimental subjects were assigned to

either an isolation- or group-reared condition (i.e., reared at 24-60 days old) and were introduced to one 90-minute feeding period per day with 22.5 hours of access to wheel activity. Results showed a higher incidence of a 25% weight loss (57% versus 18%) in rats of the isolation-reared group. There was an indication of slight atrophy of the thymus glands in the rats of the group-reared group. However, researchers did not find any indication of gastric ulcers or any differences in the adrenal weights between the two groups (Ness et al., 1995).

Hormones

Another component to take into consideration is the regulation of hormonal secretion from endocrine glands as it is related to diet and stress. It has been well indicated that stress, whether mental, physical, or any stimulant of the pituitary-adrenal axis, induces the secretion of corticotropin-releasing hormone (CRH). Increased levels of CRH stimulate adrenocorticotrophic hormone (ACTH) release from the anterior pituitary glands, and elevated levels of ACTH ultimately stimulate the adrenal cortex for the production of cortisol or corticosterone, all of which are regulated via negative feedback inhibition. However, in patients with anorexia nervosa, there is an increase in CRH and cortisol, however, the stimulation of ACTH from CRH is blunted because researchers found either normal levels or lower levels of ACTH in AN patients (Licinio, Wong, & Gold, 1996).

Cortisol counteracts the roles of insulin by inhibiting the activation of glycogen synthase and the inhibition of glucose production and lipolysis (J. E. Hall & Adair, 1998). Past studies have shown that if cortisol levels are elevated food intake should be suppressed (Licinio et al., 1996; Rivier & Plotsky, 1986). For example, the effects of

activity on food consumption in rats injected with either saline or alpha-helical corticotropin-releasing factor (CRF), which is a CRF antagonist, in the right lateral ventricle of the brain were studied. Researchers found that increases in CRF levels were proportional to the risk of anorexia; that is, animals injected with saline and exposed to forced physical activity (i.e., 40min motor-driven treadmill) demonstrated slower growth and reduced food intake compared to those injected with saline without exposure to forced activity. However, treatment with the CRF-antagonist prevented this anorexic effect probably by hindering the decrease in food consumption and bodyweight in exercising animals (Rivest & Richard, 1990).

In addition, researchers have shown that underweight anorexic patients display elevated levels of CRH in the cerebrospinal fluid (CSF), which is then normalized after weight restoration (Kaye et al., 1987). Similarly, anorexic patients show low responses to elevated plasma cortisol levels, yet demonstrated normalization of levels and responses after weight recovery. In healthy individuals, high levels of cortisol signal negative feedback inhibition to decrease cortisol production and an increase in cortisol metabolism in order to normalize plasma levels. Because the response (i.e., increased cortisol metabolism and decreased cortisol production) is not high in anorexic patients, the result is sustained hypercortisolism (Hotta et al., 1986). Boyar and lab first reported that the hypercortisolism was an outcome of decreased cortisol metabolism (Boyar et al., 1977).

Another study suggested that low weight and hyperactivity, which is normally seen in patients with anorexia, is not the primary causal factors in abnormal neuropeptides (e.g., cholecystokinin [CCK], CRH, neuropeptide Y [NPY], galanin [GAL], proopiomelanocortin [POMC]), rather food restriction is the principal contributor

(Wong, Licinio, Gold, & Glowa, 1993). In this study, researchers measured the gene expression levels for CCK, CRH, and POMC, which decrease food intake and NPY and GAL, which increase food intake. These neuropeptides are involved in the regulation of appetite and were measured in the paraventricular (PVN) and arcuate (ARC) areas of the brain (animals were killed 30 min after meal period). Plasma measurements of ACTH and corticosterone were measured as well. Food intake and bodyweight were also measured daily. They found that although there were no significant differences in final food intake (experimental animals ate 9% less than their controls) and final body weight, there was a significant increase in ACTH and corticosterone in the experimental animals when compared to the controls. There were no significant effects on the gene expression levels of CCK, CRH, NPY, GAL, and POMC. Because food intake and body weight were not significantly different, it was suggested that activity did not have an effect on these neuropeptides even though it was associated with elevated levels of ACTH and corticosterone (Wong et al., 1993). Thus, whether the increase in CRH/CRF (i.e., cortisol) level is the causal or the resulting factor or whether reduced food intake, decreased bodyweight, or hyperactivity, is the primary or secondary factor, it is well supported in several past experiments that there is a strong association between the elevation in cortisol levels and anorexia (Hotta et al., 1986; Kaye et al., 1987; Licinio et al., 1996; Rivest & Richard, 1990; Wong et al., 1993).

Serotonin, also known as 5-hydroxytryptamine (5-HT), is another hormone that plays a role in feeding behavior. Its receptors are widely found in the hypothalamus and in the gut, thus, alterations to the hypothalamus (e.g., lesions or drug injections) or modifications of the gastrointestinal tract (e.g., increases in intraluminal pressure or pH in

duodenum) can cause changes in eating behavior. Serotonin has a specific effect on satiety rather than hunger signals (Blundell, 1977), thus, increases in serotonin levels are expected to decrease food consumption. Scientists have developed anorectic drugs (e.g., fenfluramine and fluoxetine), which act on serotonergic mechanisms to inhibit food intake, for the treatment of obesity (Carruba et al., 1986; Fessler, 2002; Rieg et al., 1994). However, the effects of elevated serotonin (5-HT) on food intake have shown conflicting results in one study. For example, researchers found that increases in serotonin levels did result in a reduction in food intake (Carruba et al., 1986). However, others have shown that low levels of serotonin levels were associated with a decreased food intake and that elevated serotonin levels resulted in a reduction in weight loss (Altemus, Glowa, Galliven, Leong, & Murphy, 1996). In the former study, researchers demonstrated that subcutaneous injections of serotonin were strong enough to suppress food intake in hyperphagic-induced rats injected with insulin or 2-deoxy-D-glucose (2-DG). In the latter study, researchers tested the effects of two drug treatments in rats: fluoxetine, which improves serotonin activity, and parachlorophenylalanine (PCPA), which is a tryptophan hydroxylase inhibitor. They found that rats treated with fluoxetine via intraperitoneal (IP) injections significantly lost less weight, showed less activity, and increased food consumption compared to saline-treated animals. Animals treated with PCPA via IP injections demonstrated opposite results from fluoxetine-treated animals when compared to saline-treated animals (i.e., significantly lost more weight, showed more activity, and reduced food consumption). Nevertheless, more evidence indicates that serotonin levels are inversely related to food intake and that anorexic individuals display lower than normal levels of 5-hydroxyindole acetic acid (5-HIAA), a serotonin

metabolite that has been found to be at above normal levels when patients have restored body weight (Rieg et al., 1994).

Catecholamines (epinephrine [E], norepinephrine [NE], and dopamine [DA]) function in the promotion and regulation of energy metabolism. E and NE are secreted by adrenal medulla in a 4:1 ratio, respectively. The functions of E and NE include stimulation of adrenergic system, increase efficiency of muscular contraction, increase rate of glycogen breakdown into glucose, & increase rate of fatty acids (FA) release from fat. Also, E (not NE) increases release of ACTH. Because cortisol exerts permissive effects on the actions of catecholamines (E and NE), the catecholamines are not as effective as they are normally when cortisol levels are low (J. E. Hall & Adair, 1998).

Exercise tends to increase plasma NE concentrations (Duncan et al., 1985; Kohno et al., 2000). Additionally, DA plays a role in maintaining high levels of physical activity and suppressing food intake; thus contributing to the anorexic effects caused by the activity-stress model (Avraham, Hao, Mendelson, & Berry, 2001; Carruba et al., 1986; Lambert & Porter, 1992; Lyons & Truswell, 1988). Researchers studied the effects of pimozide, a dopamine-D2-receptor blocker, in rats exposed to the activity-stress model and found that although it did not affect activity levels in the dark phase, it significantly reduced wheel running in the light phase resulting in an increase in number of surviving animals (Lambert & Porter, 1992).

Diet and Metabolism

Few studies have manipulated the diet in an attempt to attenuate the negative effects of deprivation and novelty stress in the activity-based anorexia paradigm. For example, the effects of pellet-form and powder-form of food on body weight, food intake,

and level of activity were examined in one study. Neither form of food eliminated starvation in rats; however, diet restriction and activity continued to affect the ABA outcomes. Wheel access did not affect weight gain in those rats that had food available ad libitum, though, it did result in starvation of more than 75% of rats who were restricted of food and allowed access to wheels (Beneke et al., 1995).

Researchers have studied the nutritional status of the individual or subject and the nutritional content of the diet as well. Scientists have shown that tyrosine injections can prevent the activity-induced elevation of 5-HT levels in the hypothalamus thereby improving food consumption in rats exposed to the activity anorexia model. The rats to be injected with tyrosine initially lost weight and decreased food intake when introduced to the activity anorexia model; however, despite the sustained increase in activity, food intake was returned to normal when compared to that of the saline-injected controls when tyrosine injections were administered (Avraham et al., 2001). In addition, Davis and associates have suggested that carbohydrates (CHO) and branch-chained amino acids (BCAA) might prevent the activity-induced increase in 5-HT concentrations. These researchers found that during prolonged exercises, fatigue was associated with elevated concentrations of 5-HT and a high ratio between 5-HT and dopamine levels; thus, lowering levels of 5-HT may decrease fatigue and sustain high energy levels (J. M. Davis, Alderson, & Welsh, 2000). Furthermore, others have suggested that a high CHO meal should have greater effects on serotonergic effects when compared to a low CHO meal since CHO have been shown to increase plasma tryptophan (Lyons & Truswell, 1988).

In another experiment, researchers fed animals either a commercial lab diet containing 4.5% fat and 51% carbohydrates or an experimental diet that is high in fat (nearly 26% fat and no carbohydrates). The rats that were fed the commercial diet ran five times more when restricted of food, and lost roughly 29% of their initial body weight, compared to the rats that were fed the high fat diet—the rats on the high fat diet increased their running at a much slower pace than those fed the commercial diet. Ninety percent of the animals fed the high fat diet survived in contrast to only 20% of the rats fed the commercial diet. It was suggested that the energy density of the high fat diet might help animals maintain a more normal body weight when exposed to the ABA model (Barboriak & Wilson, 1972). A past study by Roy and lab demonstrated that rats fed a low-energy density diet increased food intake to increase energy consumption (H. J. Roy et al., 2003). However, as mentioned above, the contents of the meal should not be the only factor to consider in ABA.

The effect of carbohydrate metabolism on blood glucose levels (BGL) has been extensively studied especially in the study of hyperinsulinemia and insulin resistance. The transient hyper- and hypo-glycemic phase, which normally occurs hours after ingestion of a moderate to high carbohydrate meal, has been shown to signal many metabolic mechanisms (i.e., anabolism and catabolism of energy stores) that are assumed to play an important role in ABA (Ettinger, 2000; Winkler & Manchester, 2000). Insulin and glucagon, both of which are secreted by the pancreas, are the two primary hormones functioning antagonistically to maintain euglycemia. Insulin, which is released in response to high BGL, serves to lower BGL by promoting glucose uptake in cells, formation of fat (lipogenesis) and formation of glycogen (glycogenesis). Glucagon,

which is released in response to low BGL or raised epinephrine levels, functions synergistically with epinephrine to raise BGL by stimulating the hydrolysis of stored glycogen (glycogenolysis) and fat (lipolysis) (J. E. Hall & Adair, 1998).

Because impaired glucose tolerance (IGT) is frequently found in anorexic human individuals (Sheldon & Young, 1938; Silverman, 1977), the effects of plasma glucagon, glucose and insulin levels before and after dietary therapy and glucose challenges were studied. Before treatment, researchers found that circulating plasma glucagon levels and plasma glucose levels were higher in AN subjects compared to the controls after the glucose meal; however, levels were suppressed after therapy. Conversely, plasma insulin levels were lower in AN than those of the controls before and after the glucose meal and remained the same after the treatment. It was concluded that IGT found in AN patients is possibly due to abnormalities in the secretion of insulin and glucagon from the pancreas, which does not completely regularize after diet therapy (Kumai, Tamai, Fujii, Nakagawa, & Aoki, 1988).

Studies have shown that insulin-induced hypoglycemia, as well as 2-deoxy-D-glucose, can raise feelings of hunger in humans (Silverstone & Besser, 1971; Thompson & Campbell, 1977). 2-deoxy-D-glucose (2-DG) is a glucose anti-metabolite or analogue that functions to inhibit glucose uptake and metabolism. 2-DG is known to induce hyperphagia in normal individuals (Frohman & Nagai, 1976); however, it has been shown to decrease hunger in anorexic human patients when compared to controls (Aravich, Stanley, & Doerries, 1995). A study with humans suggested that the paradoxical response to 2-DG is not limited to those with anorexia nervosa. The double-blind study included six participants with anorexia (ranging from 51.4 to 68.4% IBW),

three participants with bulimia (106.3%, 100%, and 51.4% IBW), and six controls participants (ranging from 85.3% to 104.2% IBW). The participants were infused with 50mg of either 2-DG in saline or saline alone over 20 minutes. For a total of three hours, participants evaluated their hunger and thirst ratings on a line scale 30 minutes before infusion and every 30 minutes after infusion. Blood, blood pressure, pulse rate, and oral temperature were taken at those times as well. Approximately 2.5 hours after the start of infusion, the participants were given a 20-minute lunch and a questionnaire to evaluate their judgment of satiety. In the end, all the anorexic participants showed a decrease in hunger ratings after the 2-DG infusion. Only the emaciated bulimic participant (51.4% IBW) showed the paradoxical effect of 2-DG while those of normal weight increased their hunger ratings (Nakai, Kinoshita, Koh, Tsujii, & Tsukada, 1987).

Consistent with the results reported for humans, in an animal study, rats were matched by weight and assigned to one of three experimental groups (deprived of food only, allowed access to wheels only, or exposed to both conditions) or the control group. When the animals lost 25% or more of their initial weight, they were randomly assigned to receive either a 2-DG or saline injection within two hours before the 6-hour feeding period and then decapitated. The 2-DG injection caused significant reductions in food intake in both the ABA animals and the animals that lost weight due to food deprivation. It was proposed that 2-DG has a paradoxical effect for underweight individuals and does not affect those at a normal or above-normal body weight (Aravich et al., 1995).

Furthermore, the effects of ABA on plasma glucose and insulin levels in animals administered 2-DG injections were examined. The protocol was similar to the one described above, except that all the groups received 2-DG injections. Results show that

weight changes were comparable to the previous experiment. In addition, plasma glucose and insulin levels were reduced in ABA subjects and subjects deprived of food when injected with 2-DG before a meal. Researchers concluded that the abnormal effects of 2-DG on appetite, plasma glucose and plasma insulin levels, which all contribute to weight loss, that occur in this model are strongly comparable to anorexia nervosa in humans (Aravich et al., 1995). Consistent with these results, Pare demonstrated that AS rats had a significant reduction in liver glycogen and serum glucose; therefore, they proposed that these results were due to “exhaustion of metabolic substrates (p.419)” (Pare, 1980).

The theory of the glycemic index (GI) of foods first gained popularity in 1981 (Berning, 2000; Brand-Miller, Nantel, Suma, & Lang, 2001). GI represents the ratio of the area under the curve (AUC) of the blood glucose response curve over the first two hours of ingesting of a given quantity of the test CHO and AUC of the blood glucose response over the first two hours of consuming the same quantity of CHO from a standard food (e.g., white bread or glucose) (Vonk et al., 2000). This ratio, or GI score, indicates the potential of the CHO to raise blood glucose levels after ingestion of that particular CHO. Foods with a high GI have been shown to have a higher overall blood glucose response during the first 2 hours after meal consumption and an elevated secretion of insulin compared to foods with a low GI.

About two decades ago, a diet/meal with a low glycemic index was found to be beneficial because it produces only a minimal rise in post-prandial glucose and insulin levels. A high-GI diet/meal would have otherwise produced varying fluctuations in blood glucose and insulin levels and eventually low BGL in the late post-prandial period (Brand-Miller et al., 2001; Crapo, Insel, Sperling, & Kolterman, 1981; Febbraio, Keenan,

Angus, Campbell, & Garnham, 2000; Jenkins et al., 1987). Low GI foods take longer to digest and absorb, thus, suppressing hepatic glucose and fatty acid production (Liljeberg, Akerberg, & Bjorck, 1999).

In one study, healthy male human volunteers were assigned to an individualized low-GI diet (test 1) and a high-GI diet (test 2) for two 2-week testing periods. The low-GI diet consisted of 26g of fiber per 1000 kcal with a GI score of 64. The high-GI diet contained 21g of fiber per 1000 kcal and had a GI score of 104. Results showed that there was a 37% smaller rise in post-prandial BGL over 12 hours for the men on the low-GI diet compared to the men on the high-GI diet. In addition, insulin secretion, which was calculated from 24-hour urinary C-peptide measurements, was 32% lower in the low-GI diet. At the end of the high-GI and low-GI periods, results from another standard test meal demonstrated higher blood glucose levels 45-minutes following the low-GI diet, although C-peptide levels remained low. It was concluded that a low-GI diet has a positive effect on insulin secretions, and therefore CHO and lipid metabolism, in healthy male humans (Jenkins et al., 1987). Likewise, in another study, a high GI meal produced higher post-prandial BGL, however, it also created lower BGL at the onset of exercise compared to a low GI meal despite no differences in exercise performance (Febbraio et al., 2000). Conversely, other studies have shown a different effect (Brand-Miller et al., 2001; Kirwan, Cyr-Campbell, Campbell, Scheiber, & Evans, 2001; Thomas, Brotherhood, & Brand, 1991). In these trials, the low GI theory (i.e., the slow release of glucose into blood) has been shown to enhance physical performance, for example, by increasing exercise endurance if the meal was consumed 45 minutes to 1 hour rather than 3 hours prior to exercise (Brand-Miller et al., 2001). Perhaps the difference between the

two studies is the method of measurement—the former study measured exercise performance within 120 minutes while the latter studies measured performance via a fatiguing concept (i.e., participants are evaluated based on the amount of time it takes to reach exhaustion).

Recently, resistant starch (RS) has gained attention for possessing characteristics that are similar to dietary fiber, especially to that of soluble fiber. It has been reported to produce BGL responses similar to that of low GI foods (Jenkins et al., 1987; Ring, Gee, Whittam, Orford, & Johnson, 1988). RS, which is found at low amounts in many foods, is the form of starch that escapes the small intestine and is primarily fermented in the large intestine, which results in decreased post-prandial glucose and insulin response. RS can be defined as “the sum of starch and products of starch hydrolysis not absorbed in the small intestine of healthy individuals (p. 23)” [from (Muir, Young, & O'Dea, 1994): European Concerted Action of Resistant Starch (EURESTA), June 1990]. RS has been categorized into four types: RS₁, RS₂, RS₃, and RS₄; however, most interest has been directed to RS₂ and RS₃ (Muir et al., 1994). RS₂ is the ungelatinized starch granule, which is unavailable to digestible enzymes (e.g., amylase) due to its compact, unhydrated structure. This type of RS can be found in cereals, legumes, raw potatoes, unripe bananas, or high amylose maize starch and is most commonly used in research. RS₃ consists highly of crystalline amylose and amylopectin, which forms during starch retrogradation or recrystallization. This type of RS can be found in bread or cooled boiled potatoes since it forms after the heating and later cooling of the food product (Haralampu, 2000; Muir et al., 1994).

In an experiment that examined the digestion of resistant starch in the small intestine, researchers studied the metabolism of highly digestible cornstarch (DCS), Hylon VII (RS₂) and Novelose 330 (RS₃). Twenty-one healthy human volunteers consumed 40g of glucose and one of the three starches after fasting for 10 hours. Measurements of plasma exogenous glucose concentrations and breath excretion of ¹³CO₂ are indirect indications of intestinal digestion. Analysis revealed the highest response in breath hydrogen and exogenous glucose response levels after the DCS meal, primarily because of its high digestibility. Average GI scores, which were determined after the intake of 40 grams of starch, were 98±138, 22±13, and 48±28 for DCS, Hylon VII and Novelose 330, respectively. DCS was estimated to be 80% digestible in contrast to an approximate 50% digestibility of the resistant starches, with no differences between the two types (Vonk et al., 2000).

Because resistant starch is fermented in the large intestine, the production of short-chained fatty acids (SCFA) (e.g., butyric, acetic, and propionic acids) may contribute to “total metabolizable energy” (Behall & Howe, 1996; Cummings & Englyst, 1987; Ferguson, Tasman-Jones, Englyst, & Harris, 2000). Twenty-four volunteers (ten controls and fourteen hyperinsulinemic participants) were fed individualized diets with the primary carbohydrate as either high amylose cornstarch (AM: containing 70% amylose, 30% amylopectin) or a standard cornstarch (AP: containing 30% amylose, 70% amylopectin) for fourteen weeks. Although results showed that the consumption of the AM diet resulted in increased fecal output and nitrogen, there were no differences in metabolizable energy between the two diets. The results indicated that utilization of RS

provided energy for both the control participants (81.8% utilization) and for the hyperinsulinemic participants (53.2% utilization) (Behall & Howe, 1996).

There are implications that resistant starch may have a positive effect for overweight, diabetic, hyperlipidemic, or cancerous individuals (Raben, Tagliabue et al., 1994); (Muir et al., 1994). Evidence have supported the idea that RS can help regulate the release of anabolic and catabolic hormones by producing minimal fluctuations in the blood glucose level after a meal (Byrnes, Miller, & Denyer, 1995; Hallfrisch & Behall, 2000). The slow absorption of RS in the small intestines of humans caused reductions in post-prandial glucose and insulin levels (Muir et al., 1994). The effect on post-prandial plasma concentrations of glucose, lipids, and hormones in normal-weight, young males were studied. Participants were assigned either to a diet containing 54.1% RS (R-meal) or to one that is 100% digestible (S-meal) for two test periods. Results showed that plasma glucose, lactate, insulin, GIP, and GLP-1 levels increased markedly after consumption of the S-meal compared to R-meal. Plasma triglycerides decreased after both S-meals and R-meals. Plasma glycerol also decreased after the S-meal, but remained reasonably constant after the R-meal. Significant differences were not found in norepinephrine levels after both meals, but epinephrine levels increased after the S-meal (Raben, Tagliabue et al., 1994).

Summary

In summary, it can be concluded that:

1. Excessive exercising, diet restriction, and low body weight is strongly associated with anorexia.

2. The results of the activity-based anorexia model mimic the characteristics of individuals with anorexia nervosa.
3. Animals exposed to the ABA procedure typically display reduced food intake and body weight, increased activity, elevation of corticosterone levels, hypertrophy of the adrenal glands, and atrophy of the thymus glands.
4. Young animals weighing less than 250g (on average) are more prone to the ABA paradigm; however, it is controversial whether which gender is more vulnerable to the ABA model.
5. Pre-adaptations to the feeding schedule or activity schedule, changes in the feeding duration, frequency, and time of day, and variations in the access to the running wheels can affect the ABA outcomes.
6. Elevated levels of CRH, ACTH, corticosterone, epinephrine, norepinephrine, and/or dopamine may be associated with anorexia or cachexia.
7. A high-fat diet provides protection for animals exposed to the ABA model compared to a normal commercial diet.
8. Incorporation of resistant starch in the diet can result in significant reductions in post-prandial glycemia and insulinemia, therefore suppressing hepatic glucose and fatty acid production and conserving energy stores (i.e., glycogen and fat) in the body.

CHAPTER 3

MATERIALS AND METHODS

The activity-based anorexia (ABA) animal model has clinical significance because many individuals with anorexia nervosa often use intense exercising and severe diet restriction as a means of losing weight and maintaining the weight loss (Kron et al., 1978). To our knowledge, only a few experiments have directly examined the effects of diet composition on activity-based anorexia. Therefore, the proposed study was conducted in two experiments. Experiment one was done to replicate the standard ABA procedure using a control diet (see Table 1). Experiment two included resistant starch (RS₂), which has a lower glycemic index, in the experimental diets (see Table 1) during exposure to the activity-based anorexia paradigm. Resistant starches, which have recently gained interest for its similarity to dietary fiber, are glucose polymers that resist digestion by enzymes in the small intestines; consequently, the products of hydrolyzed starch are not readily absorbed in the small intestine. Muir and associates demonstrated that the slow absorption of RS in the small intestines of humans caused reductions in post-prandial glucose and insulin (Muir et al., 1994). Therefore, with the diet manipulation, it is predicted that the resistant starch diet will cause minimal rises in the blood glucose levels, thus, a slower, controlled release of anabolic and catabolic hormones (Byrnes et al., 1995; Hallfrisch & Behall, 2000; Raben, Tagliabue et al., 1994), and ultimately preserving the body's energy stores and reducing weight loss.

General Materials and Procedures

Animals. Sprague-Dawley male rats were purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). They were maintained on a 12-hour light/dark cycle

Table 1. Diet Composition¹: Percent of Diet and Caloric Breakdown

Ingredients	Experiment 1		Experiment 2			
	Control Diet		Control Diet		Resistant Starch Diet	
	Percent ²	Kcal ³	Percent ²	Kcal ³	Percent ²	Kcal ³
Amylopectin Cornstarch ⁴	46.0	1.65	53.1	1.91	19.7	0.71
High amylose starch ⁵	--	--	--	--	33.3	0.52
Casein ⁶	20.0	0.72	20.0	0.72	20.0	0.72
Sucrose ⁶	10.0	0.40	10.0	0.40	10.0	0.40
Cellulose ⁶	5.0	0.00	5.0	0.00	5.0	0.00
Mineral Mix (AIN-93G) ^{1,6}	3.5	0.03	3.5	0.03	3.5	0.03
Vitamin Mix (AIN-93G) ^{1,6}	1.0	0.04	1.0	0.04	1.0	0.04
Choline Bitartrate ⁶	0.3	0.00	--	--	--	--
Choline Chloride ⁶	--	--	0.1	0.00	0.1	0.00
L-Cystine ⁶	0.3	0.01	0.3	0.01	0.3	0.01
Soybean Oil ⁷	14.0	1.26	7.0	0.63	7.0	0.63
BHT ^{6, 8}	0.001		0.001		0.001	
Kcal/g Food		4.11		3.74		3.05

¹ AIN-93G (Reeves, Nielsen, & Fahey, 1993)

² All values are in percent of diet

³ All values are in kcal per g food

⁴ High Amylopectin starch (100% amylopectin), (Cerestar, Hammond, IN), (3.5kcal/g)

⁵ Hi-Maize® (60% amylose, 40% amylopectin), (Renford, Plover, WI), (estimated as 1.65 kcal/g)

⁶ Sucrose was attained from Thrifty Maid (Sun Mateo, CA) and Casein, Cellulose, Mineral Mix AIN-93G, Vitamin Mix AIN-93G, Choline Bitartrate, Choline Chloride, L-Cystine, and BHT were attained from Dyets (Bethesda, PA), which also provided energy values of diet ingredients.

⁷ Soybean oil (Aster) was attained from Deep South Products.

⁸ 0.015% of Butylated Hydroxytoluene (BHT) was mixed in soybean oil

in a temperature-controlled room (22-25 degrees Celsius) that was automatically illuminated at 7AM-7PM. Water was available ad libitum throughout the experiment. Unlimited access to powdered food was available to all the animals throughout the acclimation period and to specified groups (see below) during the experimental period. All diets were modified versions of the American Institute of Nutrition-93-Growing diet (AIN-93G) (Reeves et al., 1993). The compositions of the diets are shown in Table 1. The different diets and feeding schedules are described in further details below.

Apparatus. All the animals in experiment one were housed in Columbus Instruments Mechanical Activity Monitor Wheels (Columbus, OH), which are made of stainless steel and Plexiglas. The wheel, which has a mesh running surface and measures 14" in diameter and 4" in width, is connected to a mechanical counter that records the number of complete wheel revolutions in either direction. Adjacent to the wheel, but separated by a sliding door, is a 10"x 6"x 5" mesh wired-bottom cage for which the animal has access to food and water. The sliding door was manually opened and closed by the experimenter. A water bottle is mounted on the side of the cage with the mouthpiece inserted through a mesh hole, and a food cup is secured within the cage by wired springs. In experiment two, half the animals were housed in the Columbus Instruments Mechanical Activity Monitor Wheels (as used in experiment one), and the other half were housed in standard 8"x 10"x 7" hanging stainless steel cages with wired bottoms. Those housed in the hanging steel cages received water via a watering system and those housed in the mechanical wheels received water via water bottles (see below for details on housing). All food cups are secured within the cages by wired springs in both housing environments.

General Procedures. Upon arrival in the laboratory, the animals were acclimatized to the environment, and food and water were available ad libitum throughout this period. A run-test, which is an assessment of spontaneous activity, was administered in order to eliminate abnormally low- and high- level runners (details below). Epling and Pierce selected the “most active” animals and assigned them to the running groups (Epling & Pierce, 1984). However, to avoid unusually low or high activity, assignment to running groups was determined by selecting half the animals with the total number of wheel revolutions nearest to the mean number of total wheel revolutions during the run-test. The chosen animals were stratified by the results from the run-test and by their current weight and then randomly assigned into one of the running groups. The remaining animals were stratified by their current weight and randomized into one of the groups with no access to running wheels.

During the experimental period, the animals followed their specified feeding and activity schedules (more details below). Wheel rotations for the individual running animals were recorded daily and all the animals were weighed everyday. Throughout the run-test and experimental periods, the animals were not restricted to the wheels in order to avoid forcing activity upon the animals. Measurements for food intake were taken immediately before and after the feeding period for animals on a restricted feeding schedule by weighing the food cups and food spillage daily; however, measurements for animals on an ad libitum feeding schedule were taken every other day. Food spilled from the food cups of all animals were weighed and recorded. Food was replenished at least every other day and water bottles were changed daily. The experiment ended when the majority of the animals in the ABA group (i.e., animals subjected to restricted feeding

and liberal activity) have met one of the two criteria. The animals were considered to reach the anorexia criteria when the majority of the animals from the ABA group reached 75% or less of their pre-experimental body weight for 2-consecutive days. However, the animals were considered to reach the recovery criteria when the majority of the animals from the ABA group have stabilized their weight. That is, when the animal's weight on day 4 is at or above its weight on day 1 in any consecutive-4-day period. Due to the large number of animals, the termination period took place on two consecutive days. On the day of the kill, all animals chosen for decapitation are weighed at 4PM and tissue collections began at 4:30PM. Tissues and fat pads were rapidly excised, examined, and weighed. Livers were immediately frozen in liquid nitrogen and the brains were frozen on dry ice. Trunk blood was collected and immediately placed on ice. All samples were kept in -80°C until needed for analyses.

Experiment One (2x2 Factorial Design)

In this experiment, twenty-eight 4-week old male Sprague-Dawley rats were maintained on a 12-hour light/dark cycle (automatically illuminated at 7AM-7PM). All the animals were fed a 14% fat, high-GI (Control) diet throughout the experiment (see Table 1). Upon arrival in the laboratory, the animals were assigned to wheel cages with the passage to the wheels locked. The animals were acclimated for approximately one week (day -8 to day -1), throughout which water and food were fed ad libitum and access to activity wheels was not allowed. The acclimation period occurred in the same room as the experimental period. During acclimation, all the animals were subjected to a run-test. Because there were a limited number of wheels that were functioning properly, the run-test was divided into two days. Half the animals ($n=14$) were tested on day -6 at

1:30PM-3:30PM (100% light) and the other half ($n=14$) were tested on day -5 at 1:00PM-3:30PM (100% light). As described earlier, the animals were assigned to the running groups by selecting half the animals with the total number of wheel revolutions nearest to the mean number of total wheel revolutions during the run-test. The chosen animals were stratified by the results from the run-test and by their current weight and then randomly assigned into one of the running groups. The remaining animals were stratified by their current weight and randomized into one of the groups with no access to running wheels. The four groups consisted of two running groups (R/RF, R/AL) and two non-running groups (NR/RF, NR/AL). One active group and one inactive group were fed ad libitum (R/AL, NR/AL) while the other two groups were on a restricted feeding schedule (R/RF, NR/RF; see Figure 1).

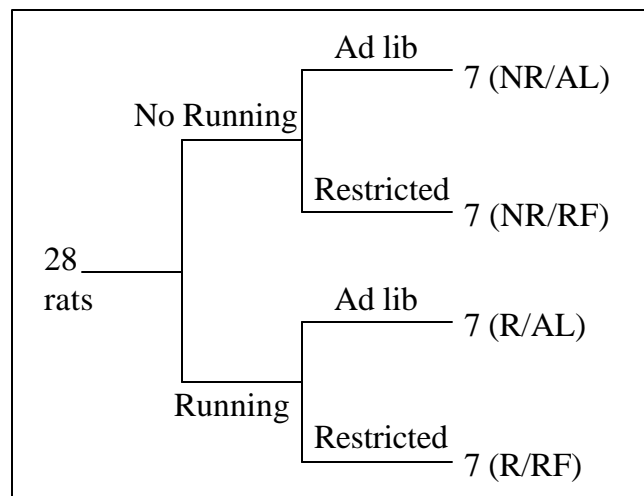


Figure 1. Experiment One ($n=28$)

At the start of the experimental period (day 0 at 6PM), the doors to the running wheels were opened for the specified animals (R/RF, R/AL) allowing them access to the

activity wheels and the food was removed from the appropriate animals (R/RF, NR/RF). From day 1 onward (see Figure 2), the restricted-fed animals were allowed one 90-minute meal period daily (4:30PM-6PM; 100% light cycle), which preceded the dark cycle, and the running animals were allowed 22 hours of activity (6PM-4PM; 55% dark/45% light cycle) immediately after the meal period. Total wheel revolutions were recorded daily at exactly 4PM, and body weights were taken during the 30-minute transition time (4PM-4:30PM), which occurred after physical activity but before the meal period. Food intake measurements were taken at least every other day throughout the experiment.

The experimental period terminated when the majority of the animals from the R/RF group reached the *recovery* criteria, that is, their weights on day 4 was at or above their weights on day 1 in a consecutive 4-day period. Blood glucose levels were measured at the time of decapitation; glucose levels were measured using the Advantage Accucheck© glucose meter from Roche Diagnostics (Indianapolis, IN). Trunk blood was collected for measurements of plasma catecholamines, corticosterone, insulin, and glucagon levels. Burden and co-authors showed an increase in corticosterone levels in experimental animals; however, they found norepinephrine levels to be consistent among all groups (V. R. Burden et al., 1993). Also, the insulin and glucose levels were significantly lower in animals exposed to both running and food-restricted conditions compared to the controls. Abdominal, perirenal, and epididymal fats, as well as brown fats, were removed and weighed. A past study (V. R. Burden et al., 1993) demonstrated that animals exposed to running wheels and restricted to one daily meal lost up to 52% body fat compared to those only food-restricted. The thymus, livers, spleens, kidneys, and adrenal glands were also removed, weighed, and examined for abnormalities.

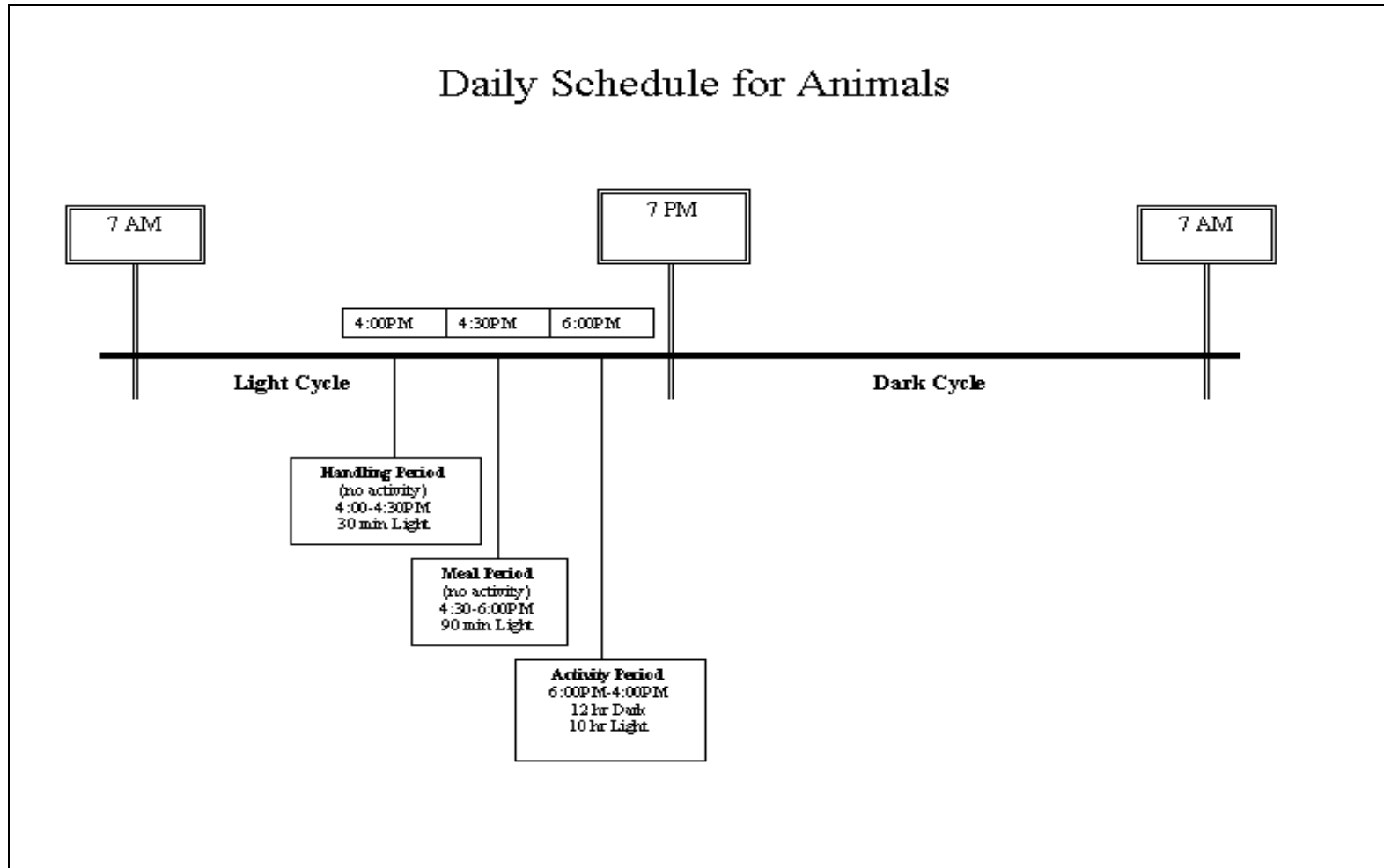


Figure 2. Daily Schedule for Animals (Experiment 1 & 2)

Studies have found a decrease in thymus and spleen weights and an increase in adrenal weights in ABA animals compared to the controls (V. R. Burden et al., 1993; Hara et al., 1981). Stomachs were removed, weighed, and examined for ulcers. The protocol was submitted to and approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University A& M College in Baton Rouge, Louisiana (Appendix A & B).

Experiment Two (2x2x2 Factorial Design)

The model used in this experiment followed the method as described above, however, there were a few modifications. In this experiment, fifty-six 4-week old male Sprague-Dawley rats were maintained on a 12-hour light/dark cycle (automatically illuminated at 7AM-7PM). Upon arrival (day -7) to the facility, the animals were randomized into the two different diet groups (see Table 1) and were fed the specified diet throughout the acclimation period, which lasted one week (day -7 to day -1), and the experimental period. All the animals were individually housed in standard hanging steel cages (as described previously) in a quarantine room for the first 5 days of acclimation (days -7 to -2) and were given water and food ad libitum. The animals were then transferred to the experimental animal room on day -2, and all animals remained in the standard cages until the start of the experiment (day 0) or unless otherwise stated below. Both the quarantine and experimental rooms were temperature-controlled and automatically illuminated at 7AM-7PM.

On the first day the rats were transferred to the experimental room (day -2), 28 rats (14 rats from the Control group and 14 rats from the RS group) were randomly selected for the first run-test. Unlike experiment one, this test allowed the rats access to

running wheels for 14 continuous hours, which consisted of 2 hours in the light cycle (5PM-7PM) and 12 hours in the dark cycle (7PM-7AM). The rats were transferred to wheel cages during the test and were returned to their standard cages when testing was not in session. At 4:30PM, the rats were transferred to the wheel cages (with their specified food and water) and the passages to the wheels were opened at 5:00PM. On the following day (day-1), the passage doors to the wheels were closed at 7AM and the rats were immediately returned to their previous cages. The same procedure followed for the second half of the animals on this day (day -1).

As in experiment one, the animals were assigned to the running groups by selecting half the animals with the total number of wheel revolutions nearest to the mean number of total wheel revolutions during the run-test. The chosen animals were stratified by the results from the run-test and by their current weight and then randomly assigned into one of the running groups within the diet group. The remaining animals were stratified by their current weight and randomized into one of the groups with no access to running wheels within the diet group. Because the animals were acclimated on a specified diet and needed to remain on the diet for the remainder of the experiment, they were stratified and randomized within their own diet group. The eight groups in this experiment consisted of four running groups (R/AL, R/RF, rsR/AL, rsR/RF) and four non-running groups (NR/AL, NR/RF, rsNR/AL, rsNR/RF). Two active groups and two inactive groups were fed ad libitum (NR/AL, R/AL, rsNR/AL, rsR/AL) while the remaining four groups were on a restricted feeding schedule (NR/RF, R/RF, rsNR/RF, rsR/RF). The animals were also fed one of two different diets. Four groups (NR/AL,

NR/RF, R/AL, R/RF) were fed a high-GI diet containing regular cornstarch (7% fat, Control) and the other four (rsNR/AL, rsNR/RF, rsR/AL, rsR/RF) were fed a low-GI diet containing resistant starch (7% fat, RS) diet (see Table 1 and Figure 3). After the assignment of groups, the animals were then transferred to their appropriate cages for the experimental period.

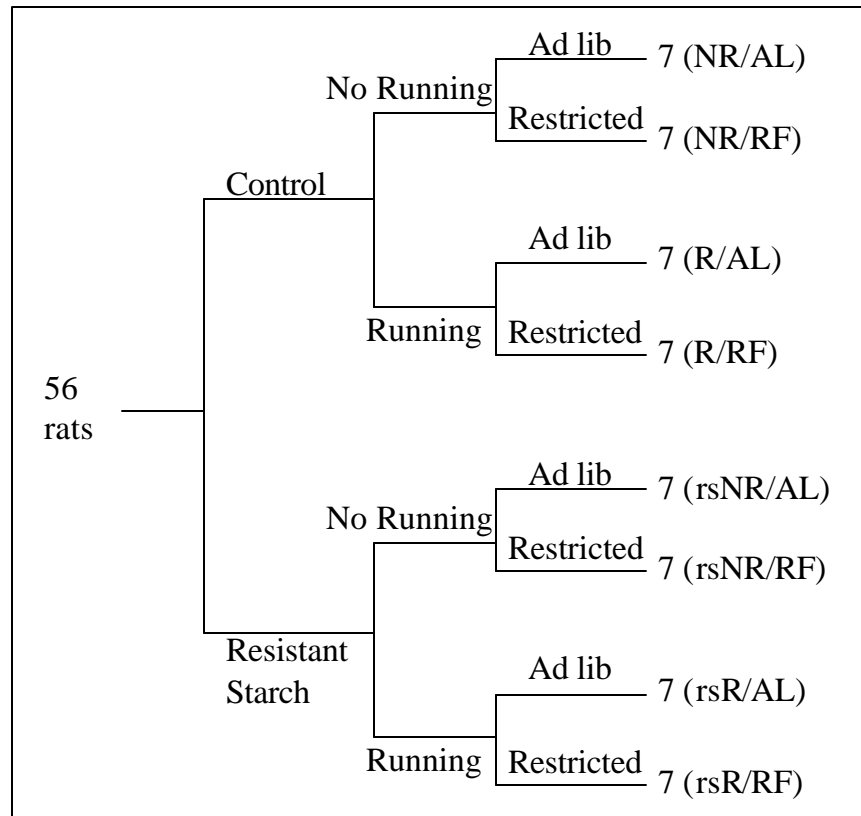


Figure 3. Experiment Two ($n=56$)

On the first day of the experiment (day 0), all animals were placed in either the standard cages or wheel cages according to their assigned group at 4PM and the daily routine for all 8 groups followed the same procedures as in experiment one. At exactly 6PM (beginning of experimental period), food was removed from those on the food-

restricted feeding schedule (NR/RF, R/RF, rsNR/RF, rsR/RF), and passages to wheels were allowed for those allowed physical activity (R/AL, R/RF, rsR/AL, rsR/RF). Food was also replenished, weighed and given to those on an ad libitum diet schedule (NR/AL, R/AL, rsNR/AL, rsR/AL) at this time.

The experiment terminated on day 5 when majority of the animals in rsR/RF group reached the anorexia criteria, that is, they experienced 25% weight loss from pre-experimental body weight. The termination period followed similar procedures as experiment one, however, there were a few changes. One, stomachs were collected without emptying and examining for irregularities; thus, we will eliminate stomach data all together. Two, blood serum was collected for two animals that were killed earlier that day (11AM) and blood plasma were collected for the other 53 animals killed later that day and the following day, however, this will not affect certain humoral analysis. Three, rather than using a glucose meter to measure blood glucose levels at time of death as in experiment one, plasma and serum glucose levels were determined by using the Infinity™ Glucose Hexokinase Liquid Stable Reagent. Four, retroperitoneal fat pads rather than abdominal fat pads were removed and weighed.

Our past experiments have found that non-fasting plasma insulin levels are lower in animals fed the RS diet compared to the controls, and abdominal, perirenal, and brown fat pad weights are decreased in animals fed the RS diet. Epididymal fat pad weights have also been shown to decrease with a RS diet (Kishida, Nogami, Himeno, & Ebihara, 2001; Pawlak, Bryson, Denyer, & Brand-Miller, 2001). The protocol was submitted to and approved by the Institutional Animal Care and Use Committee (IACUC) of Pennington Biomedical Research Center in Baton Rouge, Louisiana (Appendix C & D).

Humoral Analysis

Trunk blood was collected in 4 mL BD Vacutainer® Plus tubes spray-dried with K₂ EDTA with Hemogard™ Closures for plasma collection (Becker, Dickinson & Co., Franklin Lakes, NJ) and 13 mL standard test tubes for serum collection at decapitation. Samples were immediately placed on ice and later centrifuged at 4000 rpm at 4°C for 20 minutes. Plasma and serum samples were stored at –80°C until use for analyses.

Catecholamine Measurements. Plasma epinephrine and norepinephrine were determined by using a reverse-phase high-performance liquid chromatography with electrochemical detection (HPLC-EC). The HPLC-EC system operated with the Coulochem II Model 5600A detector, Solvent Delivery Module Model 582 pump, Guard Cell with single channel Model 5020, and Autosampler Model 542. The solvent, which contained methanol, phosphate buffer, and ion-pairing agent, used in this system was provided in the analysis kit [solvent: CAT-A-PAHSE #45-0180; Kit: Plasma Catecholamine Analysis Kit (ESA Inc., Chelmsford, MA)]. The method for catecholamine measurement was performed as described in the methodology booklet included with the kit. The CoulArray Software (version 1.04) was used to analyze the data. All samples were analyzed in duplicates.

RIA Measurements. Radioimmunoassay (RIA) kits were used to determine plasma and serum levels of glucagon (Glucagon RIA Kit, LINCO Research Inc., St. Charles, MO), insulin (Sensitive Rat Insulin RIA Kit, LINCO Research Inc., St. Charles, MO) and corticosterone (ImmuChem™ Double Antibody Corticosterone ¹²⁵I RIA Kit, ICN Biomedicals, Inc., Costa Mesa, CA). The procedures for glucagon, insulin, and

corticosterone measurements were performed as described in the methodology booklet included with the kits. All samples were performed in duplicates.

Plasma Glucose Measurements. Plasma and serum glucose was measured with the Infinity™ Glucose Hexokinase Liquid Stable Reagent (Thermo DMA, Louisville, CO). The procedure for glucose measurement was performed as described in the methodology booklet included with the package, however, a microplate reading instrument was used to analyze multiple samples rather than an individual sample during one reading. Although the incubation time ranged from 5-25 minutes across all samples and standards, the reaction was stable because Glucose Hexokinase reagents have a final reaction mixture stability of approximately 30 minutes. In addition, linear standards were used to control for the possibility in variation due to the large incubation time range. The samples were measured in duplicates (i.e., one of each sample per plate).

Statistical Analysis

Data were analyzed using Statistical Analysis Software version 8.2 (SAS). Means \pm standard error means (\pm SEM) of all variables were computed for each of the groups. Repeated measurements of variance were used for daily wheel running with day as the repeated measure. One-way analysis of variance (ANOVA) was performed on all other factors as well as cumulative wheel running to determine whether there were significant main effects of treatment among all the groups. Observed results were considered statistically significant if the probability of chance occurrence was $p < 0.05$. When one-way ANOVA indicated statistical significance, a post-hoc Tukey's Test was used to determine significant differences among treatment means and whether significant interaction(s) occurred.

CHAPTER 4

RESULTS

The results of experiment one were flawed due to uncontrollable environmental conditions and inefficient supervision of laboratory systems (see Appendix E for further explanation); therefore, experiment one and the results of experiment one will not be mentioned hereafter. The results presented below will represent only that of experiment two.

Body Weight

Tables 2, 3 and 4 provide data for the average total change in body weight (\pm SEM) from day 0 to last day of the study. There were no significant differences in initial body weights among the eight treatment groups at the beginning of the study (data not shown; $p > 0.05$). However, by the end of the study, there was a significant difference in body weight between diet groups, between feeding groups, and between activity groups as well as a significant diet*feeding interaction. As illustrated in Figure 4, animals fed the resistant starch (RS) diet had lower body weights than those fed the control (C) diet ($p = 0.002$) throughout most of the study period. The animals on the restricted feeding (RF) schedule lost weight while the animals fed ad libitum (AL) gained weight by the end of the study ($p < 0.0001$) (Tables 2 and 3). Likewise, the exercising animals lost weight and the sedentary animals gained weight by the end of the study ($p = 0.001$) (Tables 2 and 3). Lastly, as shown in Figure 5, AL animals fed the RS-diet gained approximately the same amount of body weight as the AL animals fed the C-diet; however, RF animals of the RS-diet lost 2x more weight than RF animals of the C-diet ($p = 0.002$).

Food Intake

Ad Libitum Feeding. Table 2 provides data on caloric intake and food intake expressed as mean cumulative (day 0-4) kilocalories (kcal) per kilogram (kg) of body weight (\pm SEM) and mean cumulative (day 0-4) grams (g) per kg of body weight (\pm SEM), respectively, for AL animals. On day 0-1, diet and activity had a significant main effect on caloric intake for AL animals (data not shown). Animals fed the RS-diet consumed 15% less kilocalories than those fed the C-diet ($p=0.002$). Exercising animals consumed 12% fewer kilocalories than the sedentary animals ($p=0.008$). However, on day 0-1, only activity had a significant main effect on food intake (data not shown). Exercising animals consumed 12% less food than the sedentary animals ($p=0.01$).

On day 3-4, diet and activity continued to have a significant main effect on caloric intake for AL animals (Table 2). Animals fed the RS-diet still consumed 15% less kilocalories than those fed the C-diet ($p<0.0001$); however, exercising animals only consumed 8% fewer kilocalories than the sedentary animals ($p=0.03$). Nevertheless, diet did not have a main effect on food intake, as did activity (Table 2). Exercising animals consumed nearly 8% less food when compared to sedentary animals ($p=0.02$).

Restricted Feeding. Table 3 provides information for caloric intake and food intake expressed as mean cumulative (day 0-4) kcal/kg of body weight (\pm SEM) and mean cumulative (day 0-4) g/kg of body weight (\pm SEM), respectively, for RF animals. On day 1, diet and activity did not have a significant main effect on food consumption (kcal and grams) for RF animals (data not shown). However, on day 4, there were significant main effects of diet and activity as well as a significant diet*activity interaction for caloric intake in animals on the RF schedule. Animals fed the RS-diet consumed 16% fewer

Table 2. Body Weight Change and Cumulative Food Intake (Ad Libitum)

	Ad Libitum			
	Control		Resistant Starch	
	Non-runners	Runners	Non-runners	Runners
Total Body Weight Change (g)	42.1 \pm 2.3	30.3 \pm 2.3	38.8 \pm 2.9	33.2 \pm 3.3
Cumulative Food Intake (g/kg)	395 \pm 10 ^a	347 \pm 12 ^b	396 \pm 9 ^a	384 \pm 16 ^{a,b}
Cumulative Food Intake (kcal/kg)	1486 \pm 44 ^a	1312 \pm 48 ^{a,b}	1202 \pm 38 ^b	1162 \pm 54 ^b
Note: Total body weight change represents weight change from day 0 to day of kill; cumulative data represents day 0 to day 4 (thereafter, inanition interferes with accuracy of representation); values are expressed as mean \pm SEM; significant differences at p<0.05 are indicated by ^{a, b} (Tukey-Kramer Test); n=7				

Table 3. Body Weight Change and Cumulative Food Intake (Restricted Feeding)

	Restricted Feeding			
	Control		Resistant Starch	
	Non-runners	Runners	Non-runners	Runners*
Total Body Weight Change (g)	(16.5) \pm 3.1 ^a	(30.8) \pm 3.3 ^a	(36.6) \pm 2.6 ^a	(66.1) \pm 15.4 ^b
Cumulative Food Intake (g/kg)	167 \pm 12 ^{a,b}	178 \pm 10 ^{a,b}	205 \pm 14 ^a	145 \pm 10 ^b
Cumulative Food Intake (kcal/kg)	625 \pm 44 ^a	636 \pm 22 ^a	616 \pm 42 ^a	444 \pm 30 ^b
Note: Total body weight change represents weight change from day 0 to day of kill; cumulative data represents day 0 to day 4 (thereafter, inanition interferes with accuracy of representation); values are expressed as mean \pm SEM; () indicates weight loss; significant differences at p<0.05 are indicated by ^{a, b} (Tukey-Kramer Test); n=7 unless otherwise specified; *n=6				

Table 4. Body Weight Change, Cumulative Wheel Running, and Cumulative Food Intake (Runners)

	Runners			
	Control		Resistant Starch	
	Ad Libitum	Restricted-Fed	Ad Libitum	Restricted-Fed*
Total Body Weight Change (g)	30.3 \pm 2.3 ^a	(30.8) \pm 3.3 ^b	33.2 \pm 3.3 ^a	(66.1) \pm 15.4 ^c
Cumulative Wheel Running (revolutions)	5039 \pm 392 ^a	14290 \pm 1331 ^b	7612 \pm 910 ^a	18677 \pm 2445 ^b
Cumulative Food Intake (g/kg)	347 \pm 12 ^a	178 \pm 10 ^b	384 \pm 16 ^a	145 \pm 10 ^b
Cumulative Food Intake (kcal/kg)	1312 \pm 48 ^a	636 \pm 22 ^b	1162 \pm 54 ^a	444 \pm 30 ^c
<p>Note: Total body weight change represents weight change from day 0 to day of kill; cumulative data represents day 0 to day 4 (thereafter, inanition interferes with accuracy of representation); values are expressed as mean \pm SEM; () indicates weight loss; significant differences at $p < 0.05$ are indicated by ^{a, b, c} (Tukey-Kramer Test); $n=7$ unless otherwise specified; *$n=6$</p>				

kilocalories than those fed the C-diet ($p=0.01$). Exercising animals consumed approximately 13% fewer kilocalories than the sedentary animals ($p=0.03$). As demonstrated in Figure 6, there were no significant differences between sedentary and exercising animals fed the C-diet ($p=1.0$). However, exercising animals fed the RS-diet consumed 28% less kilocalories than the sedentary animals ($p=0.02$). Exercising animals fed the C-diet ate approximately 30% more kilocalories than the exercising animals fed the RS-diet ($p=0.004$).

On the other hand, there was only a significant main effect of activity and a significant diet*activity interaction for food intake for animals on the RF schedule.

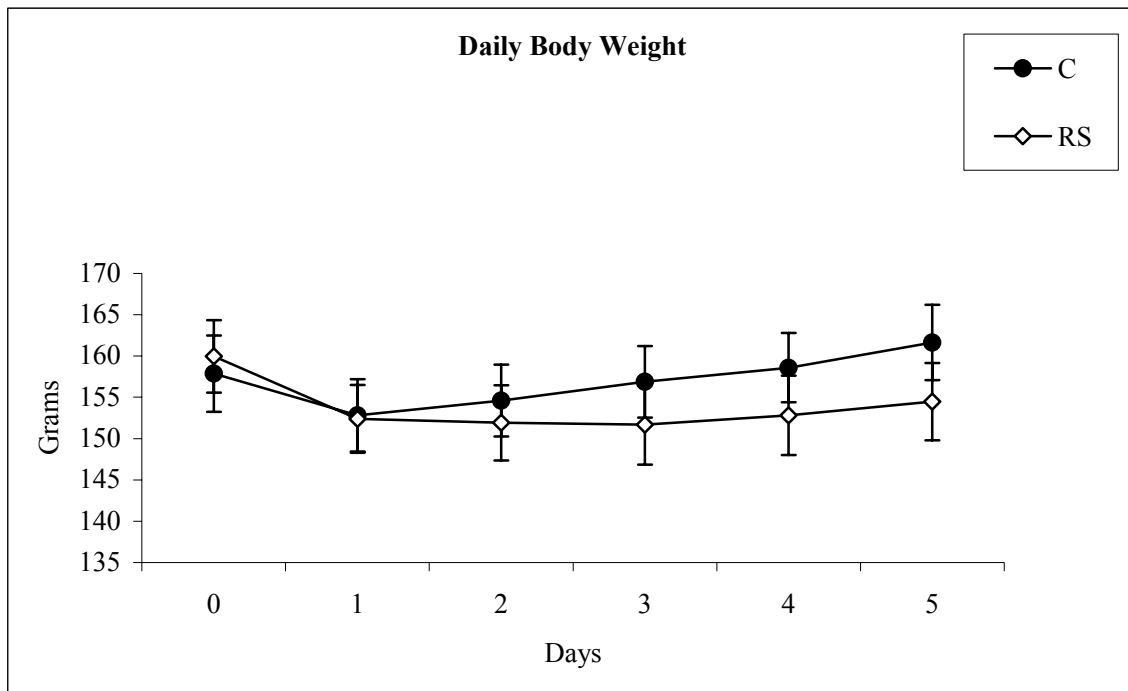


Figure 4. Daily Body Weight (Control vs. Resistant Starch diet)

Note: C=control diet; RS=resistant starch diet; day 0 represents the start of the experiment; values represent mean g per day \pm SEM

Exercising animals consumed 13% less food than the sedentary animals ($p=0.04$). As shown in Figure 7, there were no differences in food intake between sedentary and exercising animals fed the C-diet ($p=0.9$); however, exercising animals fed the RS-diet ate roughly 29% less food than the sedentary animals ($p=0.006$). There were no differences of food consumption between ABA animals of each diet group ($p=0.21$).

Activity. Table 4 provides information for caloric intake and food intake in mean cumulative (day 0-4) kcal/kg of body weight (\pm SEM) and mean cumulative (day 0-4) g/kg of body weight (\pm SEM), respectively, for exercising animals. On day 3-4, there

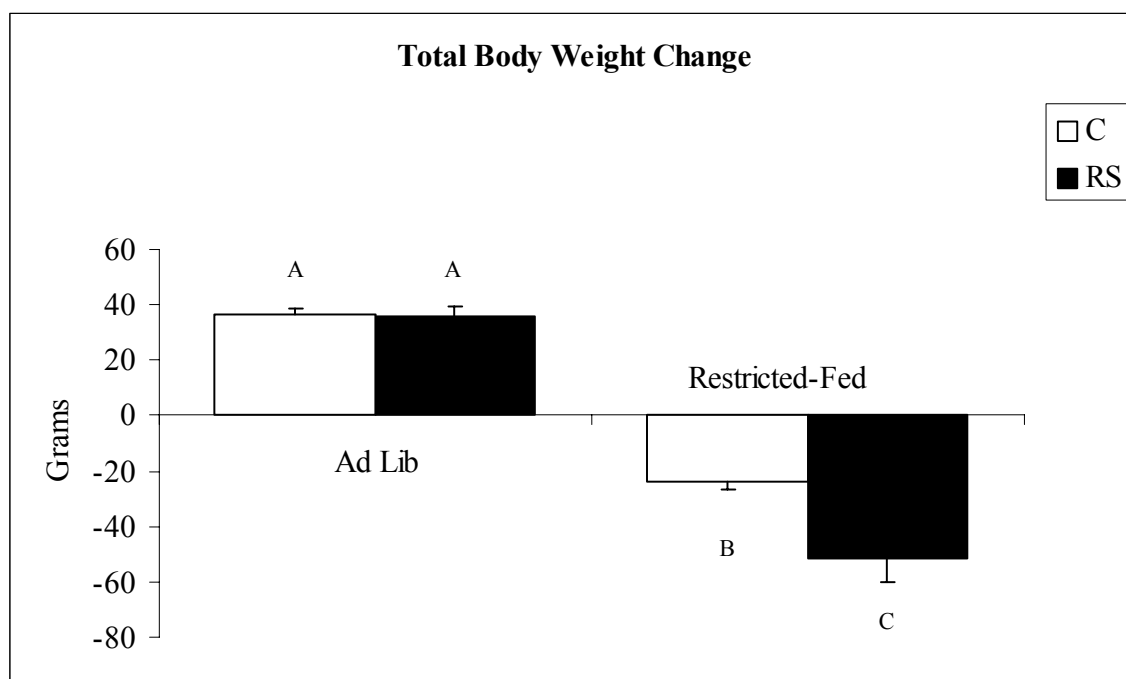


Figure 5. Total Body Weight Change (Diet x Feeding)

Note: C=control diet, RS=resistant starch diet; significant differences (Tukey-Kramer Test; $p < 0.05$) are represented by A, B, C; values represent mean grams \pm SEM

were significant main effects of diet and feeding schedule on caloric intake for exercising animals. Animals fed the RS-diet consumed 18% less kilocalories than those fed the C-diet ($p=0.0003$). Also, animals on the RF schedule consumed 56% fewer kilocalories than those AL-fed ($p < 0.0001$).

Additionally, on day 3-4, there was a significant main effect of feeding and a significant diet*feeding effect on food intake for exercising animals. Animals on the RF schedule consumed 56% less food than those AL-fed ($p < 0.0001$). As shown in Figure 8, AL-fed animals fed the C-diet consumed 10% less food than AL-fed animals fed the RS-

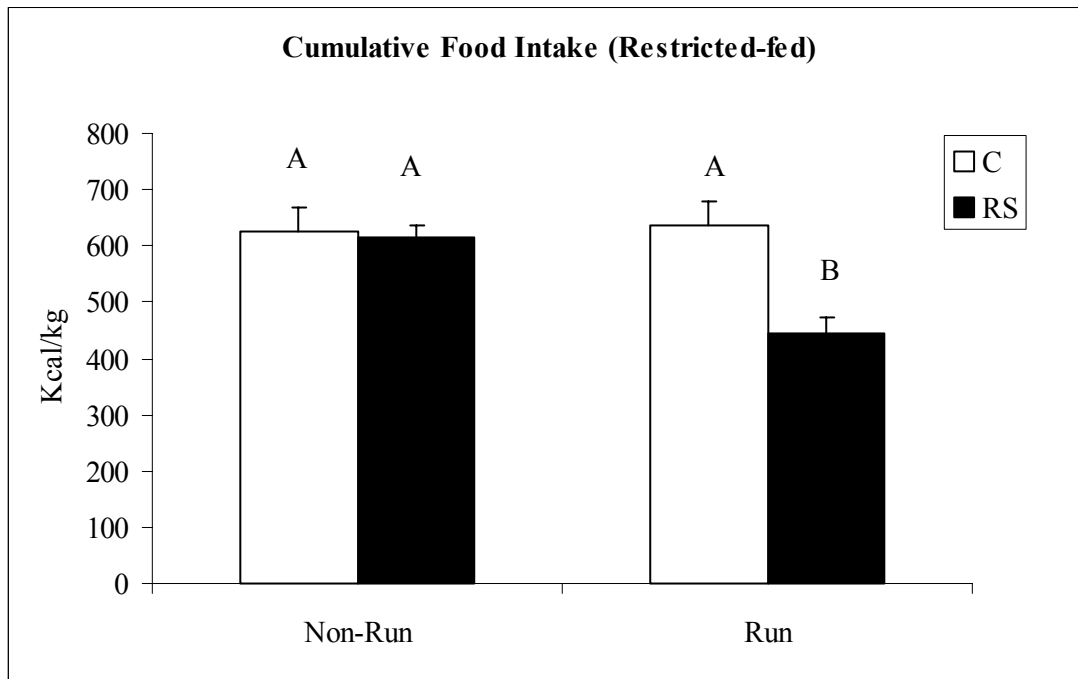


Figure 6. Cumulative Food Intake for RF in kcal/kg (diet x activity)

Note: C=control diet, RS=resistant starch diet, RF=restricted feeding; significant differences (Tukey-Kramer Test; $p < 0.05$) are represented by A and B; this illustration represent animals on the restricted feeding schedule; values are expressed as mean cumulative Kcal/kg \pm SEM

diet; however, RF animals fed the C-diet consumed 18% more food than RF animals of the RS-diet ($p=0.009$).

Wheel Running

Table 4 provides information for the average cumulative wheel running revolutions (\pm SEM) from day 0 to day 4 of the study. Throughout the study period, there was a significant main effect of diet and feed on wheel running. On day 0-1, animals fed the RS-diet ran 1.4x more than those fed the C-diet ($p=0.005$) (Figure 9a). Also, RF

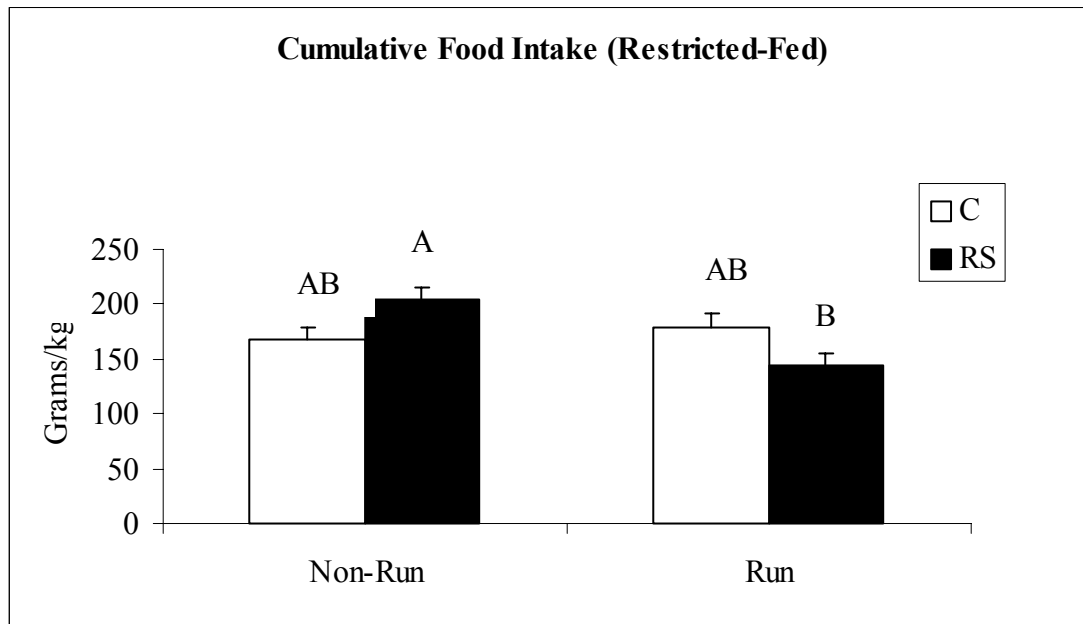


Figure 7. Cumulative Food Intake for RF in g/kg (diet x activity)

Note: C=control diet, RS=resistant starch diet, RF= restricted feeding; significant differences (Tukey-Kramer Test; $p < 0.05$) are represented by A and B; this illustration represent animals on the restricted feeding schedule; values are expressed as mean cumulative g/kg \pm SEM

animals ran 1.4x more than those fed AL ($p = 0.005$) (Figure 9b). By day 3-4, animals fed the RS-diet continued to run approximately 1.4x more (daily and cumulatively) than those fed the C-diet ($p = 0.03$) (Figure 9a); however, the animals on the RF schedule ran 2.6x more (daily and cumulatively) than those fed AL ($p < 0.0001$) (Figure 9b).

Figure 10 illustrates the running pattern for days 0-4 that the animals demonstrated during the study period. By day 3-4, the animals on the AL feeding schedule of both the RS-diet and C-diet did not significantly increase their daily activity since day 0-1 ($p = 1.00$); however, animals on the RF schedule in both diets increased their daily wheel running significantly when compared to activity on day 0-1 ($p < 0.05$), with

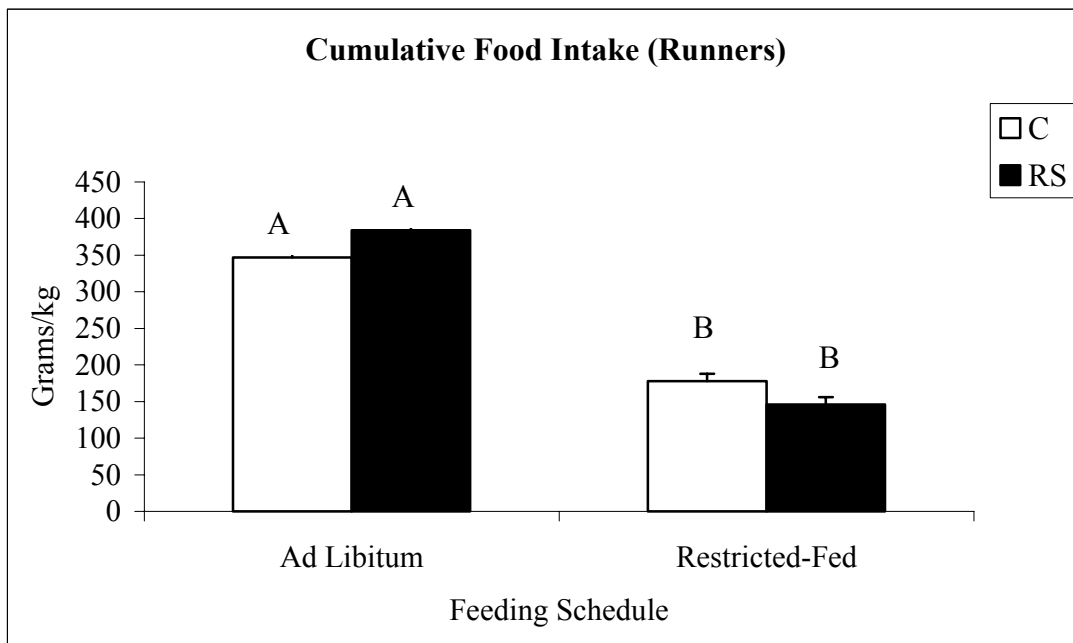


Figure 8. Cumulative Food Intake for Runners in g/kg (diet x feeding)

Note: C=control diet, RS=resistant starch diet; significant differences (Tukey-Kramer Test; $p < 0.05$) are represented by A and B; this illustration represents exercising animals; values are expressed as mean cumulative g/kg \pm SEM

the ABA animals fed the C-diet and RS-diet significantly increasing their activity as early as day 2-3 and 1-2, respectively ($p=0.002$ and $p<0.0001$).

Tissue Weights and Fat Pad Weights

Thymus, Spleen, and Liver. Tables 5 and 6 provide data of the mean weights (\pm SEM) for thymus, spleen, and liver expressed in grams per kilogram of body weight. There was a significant main effect of diet, feeding schedule, and activity on the thymus, spleen, and liver weights. Animals fed the RS-diet had 1.3x, 1.2x, and 1.2x lower thymus, spleen, and liver weights, respectively, compared to the animals fed the control diet ($p<0.0001$). The RF schedule decreased the thymus, spleen, and liver weights by 2.3x, 1.7x, and 1.4x, respectively, compared to the animals fed ad libitum ($p<0.0001$).

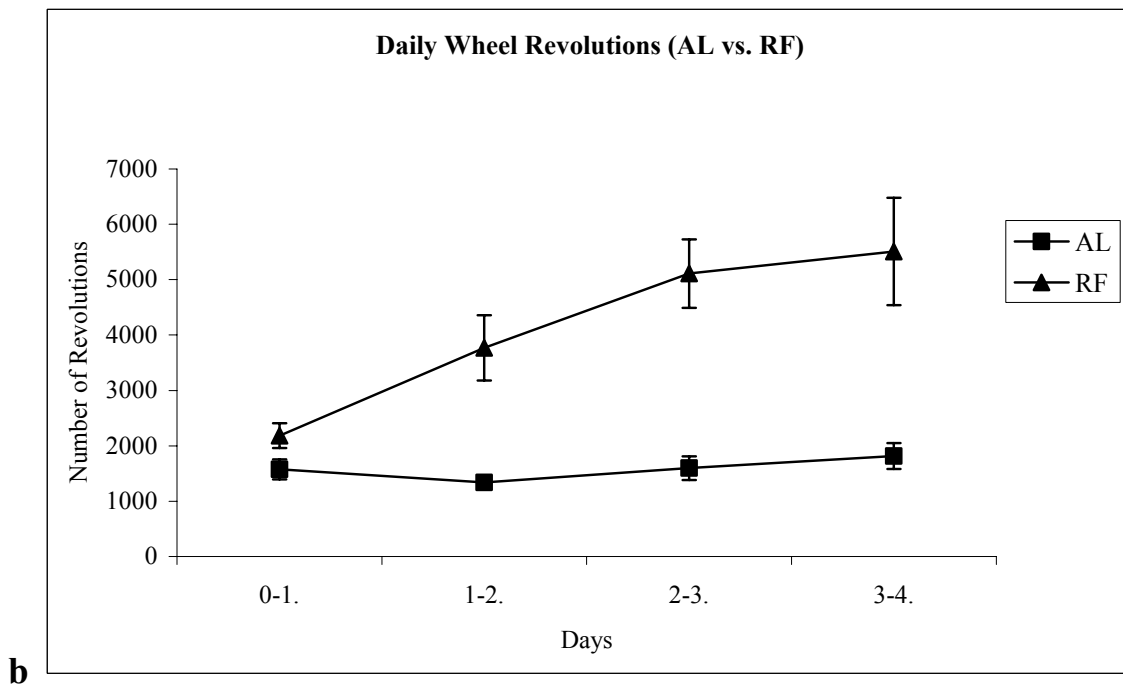
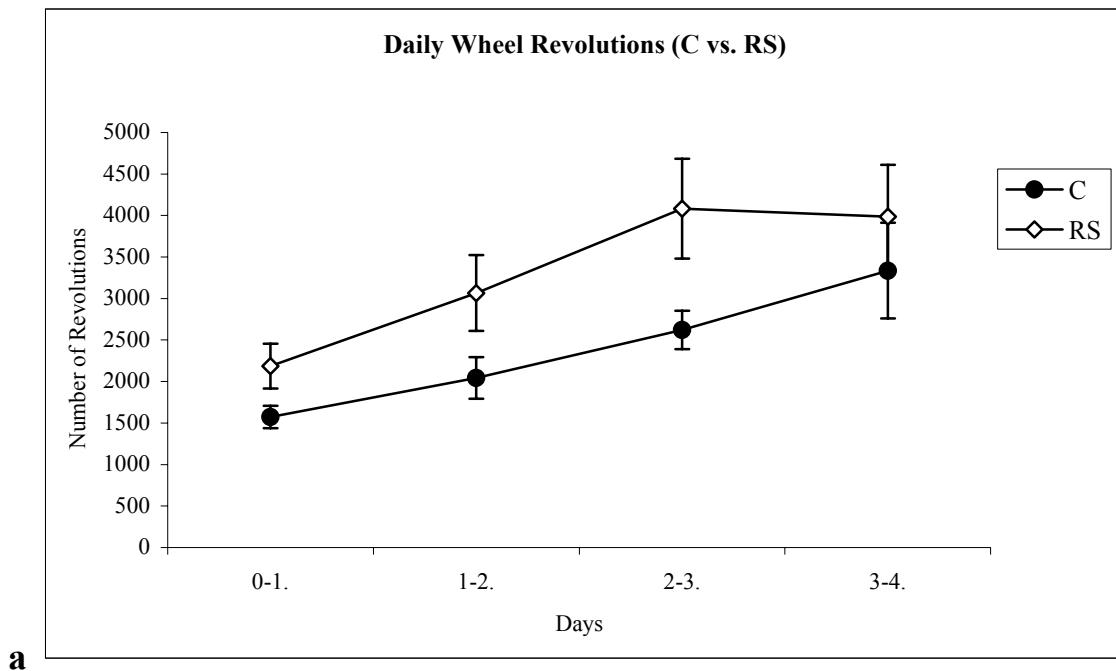


Figure 9a. Daily Wheel Revolutions (C vs. RS)

Figure 9b. Daily Wheel Revolutions (AL vs. RF)

Note: C=control diet, RS=resistant starch diet, AL=ad libitum, RF=restricted feeding; this figure represents mean daily wheel revolutions \pm SEM rather than cumulative running (see Table 4 for cumulative running data)

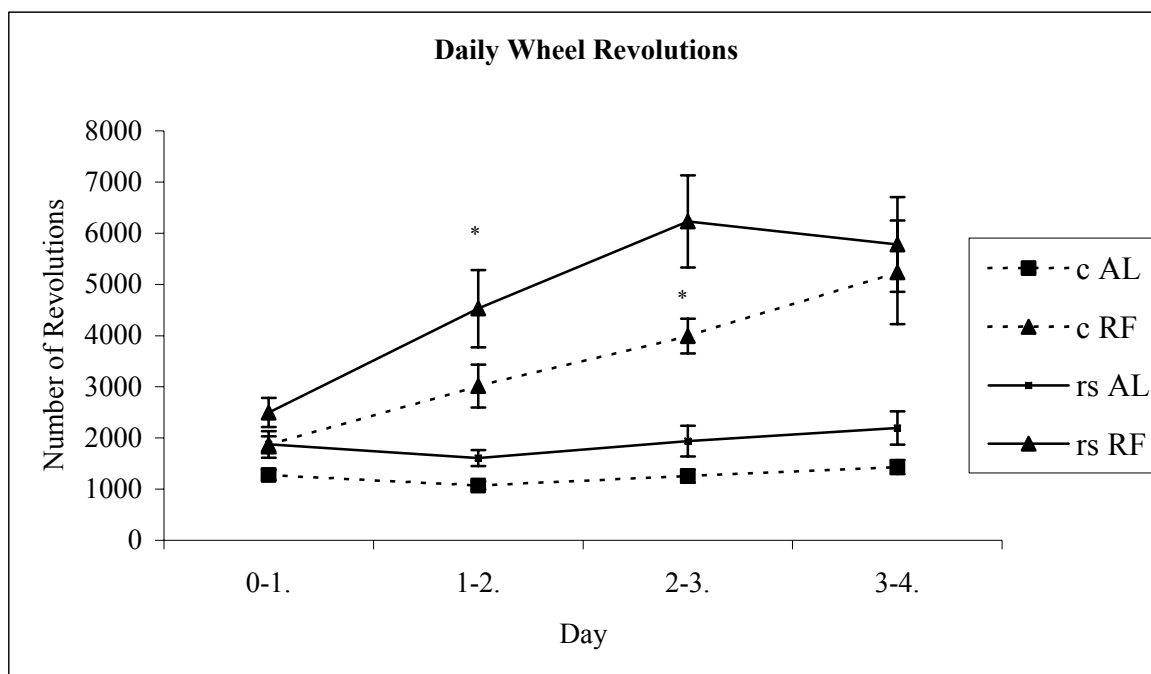


Figure 10. Daily Wheel Revolutions

Note: c=control diet, rs=resistant starch diet, AL=ad libitum, RF=restricted feeding; this figure represents mean daily wheel revolutions \pm SEM rather than cumulative running (see Table 4 for cumulative running data); * significant difference (see text)

Exercising animals had 1.2x, 1.1x, and 1.1x lower thymus, spleen, and liver weights, respectively, compared to the sedentary animals ($p=0.002$, $p=0.003$, $p=0.039$, respectively).

In addition, there was a significant diet*feeding ($p=0.002$), activity*feeding ($p=0.003$), and diet*feeding*activity ($p=0.009$) interaction for thymus weights. RF animals fed the C-diet had 1.8x lower thymus weights than those AL-fed; however, the RF animals fed the RS-diet had 3.2x lower thymus weights than those AL-fed. In animals fed AL, exercising did not greatly affect the thymus weights when compared to

Table 5. Tissues and Fat Pad Weights (Ad Libitum)

	Ad Libitum			
	Control		Resistant Starch	
	Non-Runners	Runners	Non-Runners	Runners
Thymus	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.2	3.1 ± 0.2
Spleen	3.2 ± 0.1	3.3 ± 0.1	3.1 ± 0.1	3.1 ± 0.2
Liver	38.4 ± 1.4	37.3 ± 0.8	34.0 ± 0.9	34.9 ± 1.0
Kidneys	7.9 ± 0.1	7.9 ± 0.2	7.7 ± 0.2	7.6 ± 0.2
Adrenal Glands	0.19 ± 0.03	0.19 ± 0.01	0.17 ± 0.02	0.22 ± 0.02
Retroperitoneal Fat	1.69 ± 0.35 ^a	0.80 ± 0.16 ^{a,b}	1.08 ± 0.15 ^{a,b}	0.40 ± 0.10 ^b
Epididymal Fat	6.84 ± 0.55 ^a	4.86 ± 0.28 ^{b,c}	5.58 ± 0.34 ^{a,b}	3.45 ± 0.54 ^c
Perirenal Fat	1.25 ± 0.14 ^a	0.93 ± 0.07 ^{a,b}	0.98 ± 0.07 ^{a,b}	0.69 ± 0.03 ^b
Brown Fat	1.57 ± 0.14	1.63 ± 0.08	1.40 ± 0.08	1.46 ± 0.07
Note: Values are expressed as mean g/kg ± SEM; significant differences at p<0.05 are indicated by ^{a, b, c} (Tukey-Kramer Test); n=7				

the sedentary animals (3.24g/kg vs. 3.27g/kg, respectively); conversely, in RF animals, exercising animals had 1.65x lower thymus weights than the sedentary animals. In exercising animals on the RF schedule, those fed the C-diet had 2.5x lower thymus weights than their sedentary AL-fed controls and those fed the RS-diet had 3.8x lower thymus weights than their sedentary AL-fed animals. Furthermore, in sedentary animals, animals on the RF schedule and fed the C-diet had only 1.3x lower thymus weights than the sedentary AL-fed animals, while animals on the RF schedule and fed the RS-diet had

Table 6. Tissues and Fat Pad Weights (Restricted Feeding)

	Restricted Feeding			
	Control		Resistant Starch	
	Non-Runners	Runners	Non-Runners	Runners*
Thymus	2.4 ± 0.2^a	1.3 ± 0.1^b	1.1 ± 0.1^b	0.9 ± 0.1^b
Spleen	2.5 ± 0.1^a	1.9 ± 0.1^b	$1.8 \pm 0.1^{b,c}$	1.3 ± 0.1^c
Liver	29.6 ± 0.8^a	28.2 ± 1.4^a	$25.5 \pm 0.5^{a,b}$	20.7 ± 1.4^b
Kidneys	7.7 ± 0.2	8.2 ± 0.2	7.8 ± 0.1	8.7 ± 0.4
Adrenal Glands	0.25 ± 0.02	0.26 ± 0.02	0.31 ± 0.03	0.37 ± 0.05
Retroperitoneal Fat	0.51 ± 0.43	0 ± 0	0 ± 0	0 ± 0
Epididymal Fat	1.64 ± 0.39	0.59 ± 0.19	0.57 ± 0.17	0.30 ± 0.12
Perirenal Fat	0.45 ± 0.13	0.35 ± 0.13	0.38 ± 0.10	0.09 ± 0.06
Brown Fat	1.50 ± 0.09^a	1.33 ± 0.09^a	$1.26 \pm 0.06^{a,b}$	0.90 ± 0.10^b
Note: Values are expressed as mean g/kg \pm SEM; significant differences at $p < 0.05$ are indicated by ^{a, b, c} (Tukey-Kramer Test); $n=7$ unless otherwise specified; * $n=6$				

nearly 3x lower thymus weights than the sedentary AL-fed animals. Exercising animals on the RF schedule and fed the RS-diet had nearly 1.5x lower thymus weights than those fed the C-diet, but this finding was non-significant.

There was also a significant diet*feeding ($p=0.003$) and activity*feeding ($p<0.001$) interaction for spleen weights. In the C-diet, animals on the RF schedule had 1.5x lower spleen weights than those fed AL. In the RS-diet, animals on the RF schedule had more than 2x lower spleen weights than those fed AL. In animals fed AL, the spleen

weights for exercising animals was only slightly different from the sedentary animals (3.21g/kg vs. 3.16g/kg, respectively); however, in animals on the RF schedule, the spleen weights for exercising animals was nearly 1.4x lower than the sedentary animals. There were no significant interactions for liver weights.

Kidneys and Adrenal Glands. Tables 5 and 6 provide data of the mean weights (\pm SEM) of the kidney and adrenal glands expressed as grams per kilogram of body weight. Feeding schedules had a significant main effect on both the kidneys and adrenal glands ($p=0.02$ and $p<0.0001$, respectively). In animals on the RF schedule, kidney and adrenal gland weights were 1.04x and 1.6x higher, respectively, than those AL-fed. Exercising had a significant main effect on kidney weight ($p=0.04$) but not for adrenal gland weights ($p=0.10$). Exercising animals had 1.04x higher kidney weights than those that were sedentary. On the other hand, diet only had a significant main effect on adrenal gland weights ($p=0.03$) but not for kidney weights ($p=0.91$). Animals on the RS-diet had 1.2x higher adrenal weights than those fed the C-diet.

There was a significant activity*feeding interaction for kidney weights ($p=0.02$). In animals on the RF schedule, those that exercised had 1.1x higher kidney weights than those that were sedentary. However, in AL-fed animals, both exercising and sedentary animals had similar kidney weights with the exercising animals having slightly lower kidney weights than the sedentary animals (7.73g/kg and 7.78g/kg, respectively). In addition, there was a significant diet*feeding interaction for adrenal gland weights ($p=0.03$). In the C-diet, RF animals had only 1.4x higher adrenal weights than those AL-fed. In the RS-diet, RF animals had 1.8x higher adrenal weights than those fed AL.

Retroperitoneal Fat, Epididymal Fat, and Perirenal Fat. Tables 5 and 6 provide information of the mean weights (\pm SEM) of retroperitoneal, epididymal, and perirenal fat pad weights in grams per kilogram of body weight. There was a significant main effect of diet, feeding schedule, and activity on retroperitoneal, epididymal, and perirenal fat pad weights. Animals fed the RS-diet had approximately 2x, 1.4x, and 1.4x lower retroperitoneal, epididymal, and perirenal fat pad weights, respectively, when compared to those fed the C-diet ($p=0.0179$, $p=0.0003$, and $p=0.0044$, respectively). Animals on the RF schedule had over 7.5x, 6.5x, and 3x lower retroperitoneal, epididymal, and perirenal fat pad weights, respectively, than those AL-fed ($p<0.0001$). Exercising animals had 2.7x, 1.6x, and 1.5x lower retroperitoneal, epididymal, and perirenal fat pad weights, respectively, when compared to sedentary animals ($p=0.0015$, $p<0.0001$, and $p=0.0008$, respectively).

Although there were no significant interactions for retroperitoneal and perirenal fat weights, there was a significant activity*feeding interaction for epididymal fat pad weights ($p=0.0091$). In sedentary animals, RF decreased epididymal fat weights by nearly 6x the weight of those AL-fed. In exercising animals, RF decreased epididymal fat weights by approximately 9x the fat weight of those AL-fed.

Brown Fat. Tables 5 and 6 provide data for mean brown fat pad weights (\pm SEM) in grams per kilogram of body weight. Both diet and feeding had significant main effects on brown fat pad weights. Animals fed the RS-diet had about 1.2x lower brown fat weights than those fed the C-diet ($p=0.0003$). Likewise, animals on the RF schedule had approximately 1.2x lower brown fat weights than those AL-fed ($p=0.0001$). In addition, there was a significant interaction between activity and feeding ($p=0.02$) for brown fat

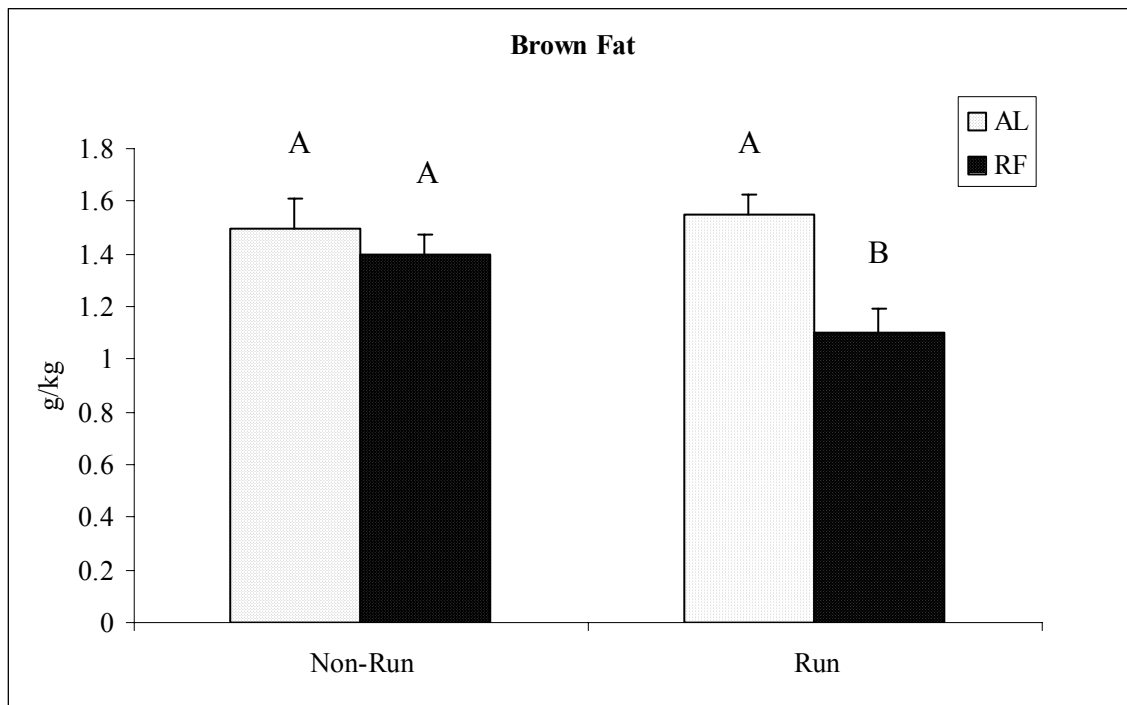


Figure 11. Brown Fat (activity x feeding)

Note: AL=ad libitum, RF=restricted feeding; significant differences (Tukey-Kramer Test; $p < 0.05$) are represented by A and B; values are expressed as mean g/kg \pm SEM

pad weights. In AL-feeding, exercising increased the weights of brown fat only slightly when compared to the sedentary animals (1.55g/kg vs. 1.49g/kg, respectively); however, in RF, exercising animals had approximately 1.2x lower weights for brown fat compared to sedentary animals (Figure 11).

Stress Hormones (Epinephrine, Norepinephrine, and Corticosterone)

Tables 7 and 8 provide information for mean epinephrine, norepinephrine, and corticosterone levels (\pm SEM). Overall, exercising and feeding schedule had a significant effect on epinephrine, norepinephrine, and corticosterone levels. Exercising animals had

Table 7. Hormones and Plasma Glucose Levels (Ad Libitum)

	Ad Libitum			
	Control		Resistant Starch	
	Non-Runners	Runners	Non-Runners	Runners
Epinephrine (ng/mL)	2.06 ± 0.26	2.40 ± 0.51	1.44 ± 0.16	2.36 ± 0.33
Norepinephrine (ng/mL)	1.08 ± 0.15	1.47 ± 0.26	0.79 ± 0.06	1.01 ± 0.12
Corticosterone (ng/mL)	791 ± 161	1000 ± 169	601 ± 106	1207 ± 154
Glucagon (pg/mL)	56 ± 8	49 ± 8	116 ± 12	92 ± 10
Insulin (ng/mL)	0.77 ± 0.08	0.96 ± 0.41	0.53 ± 0.04	0.52 ± 0.04
Plasma glucose (mg/dL)	183 ± 14	170 ± 7	179 ± 16	177 ± 14
Note: Values are expressed as mean ± SEM; significant differences considered at p<0.05 (Tukey-Kramer Test); n=7				

1.4x, 1.3x, and 1.9x more epinephrine, norepinephrine, and corticosterone levels, respectively, than sedentary animals (p<0.05, p=0.02, and p=0.02, respectively).

Additionally, those on the RF schedule had 1.5x, 1.8x, and 2.4x higher epinephrine, norepinephrine, and corticosterone levels, respectively, than those fed AL (p=0.0003, p<0.0001, and p<0.0001, respectively).

Diet did not have a significant main effect on epinephrine, norepinephrine, and corticosterone levels (p=0.25, p=0.27, and p=0.70, respectively); however, it did have a significant interaction with the feeding schedule (p=0.002) as well as with the feeding

Table 8. Hormones and Plasma Glucose Levels (Restricted Feeding)

	Restricted Feeding			
	Control		Resistant Starch	
	Non-Runners	Runners	Non-Runners	Runners
Epinephrine (ng/mL)	3.25 ± 0.73	3.27 ± 0.84	3.38 ± 1.06	6.10 ± 1.20 [^]
Norepinephrine (ng/mL)	1.75 ± 0.26 ^a	1.51 ± 0.24 ^a	1.17 ± 0.26 ^a	2.99 ± 0.57 ^{b^}
Corticosterone (ng/mL)	1705 ± 353	2373 ± 701	1777 ± 195	2647 ± 403*
Glucagon (pg/mL)	39 ± 4 ^a	73 ± 28 ^{a,b}	80 ± 11 ^{a,b}	173 ± 86 ^{b*}
Insulin (ng/mL)	0.40 ± 0.01	0.39 ± 0.01	0.38 ± 0	0.37 ± 0*
Plasma glucose (mg/dL)	140 ± 14	117 ± 14	115 ± 12	97 ± 22 [^]
Note: Values are expressed as mean ± SEM; significant differences at p<0.05 are indicated by ^{a, b} (Tukey-Kramer Test); n=7 unless otherwise specified; *n=6; ^n=4				

schedule *and* activity (p=0.022) for norepinephrine levels only. In the C-diet, animals on the RF schedule produced roughly 1.3x more norepinephrine levels than those fed AL while in the RS-diet, animals on the RF schedule produced approximately 2.6x the norepinephrine levels than the animals AL-fed. Figure 12 demonstrates a significant diet*feeding*activity interaction. In the C-diet, there were no significant differences in norepinephrine levels between exercising and sedentary animals on the RF schedule (p=0.99); however, in the RS-diet, exercising animals on the RF schedule had 1.7x higher norepinephrine levels than sedentary RF animals (p=0.04). In addition, RS-diet, exercising RF animals had nearly 4x higher levels of norepinephrine than its respective

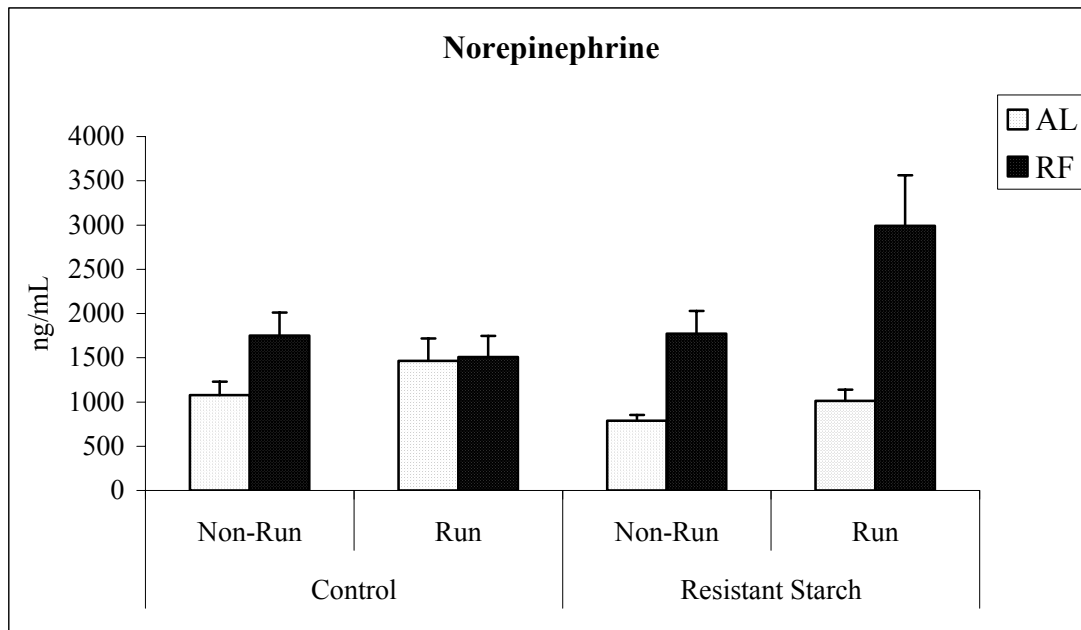


Figure 12. Norepinephrine (diet x feeding x activity)

Note: AL=ad libitum, RF=restricted feeding, C=control diet, RS=resistant starch diet; values are expressed as mean ng/mL \pm SEM

controls ($p < 0.0001$). ABA animals fed the RS-diet had nearly 2x the level of norepinephrine than the ABA animals fed the C-diet ($p = 0.007$).

Diet Hormones and Glucose

Glucagon. Tables 7 and 8 also show data for mean glucagon levels (\pm SEM). Diet had a significant main effect on plasma glucagon levels with animals on the RS-diet having 1.7x higher levels than those fed the C-diet ($p = 0.001$). Activity and feeding schedule did not have a significant main effect on glucagon levels among the treatment groups ($p = 0.25$ and $p = 0.60$, respectively); however, there was a significant activity*feeding interaction for glucagon levels ($p = 0.02$). In AL-fed animals, plasma

glucagon levels were approximately 1.2x lower for the exercising animals when compared to the sedentary animals. In RF animals, plasma glucagon levels for exercising animals were more than 2x higher than that of the sedentary animals.

Insulin and Plasma Glucose. Tables 7 and 8 show data for mean insulin and plasma glucose levels (\pm SEM). There was a significant main effect of feeding on insulin and plasma glucose levels. Animals on the RF schedule had 1.8x and 1.5x lower levels of insulin and plasma glucose levels, respectively, when compared to AL-fed animals ($p=0.006$ and $p<0.001$, respectively). Diet and activity did not have any significant main effects or interactions on insulin and plasma glucose levels ($p\geq 0.10$).

CHAPTER 5

DISCUSSION

The present study was designed to determine whether resistant starch could ameliorate the outcomes of the activity-based anorexia (ABA) model. The results of the experiment also served to validate this specific procedure as an ABA paradigm. Consistent with many previous studies, young, male rats from our study exhibited decreased food intake, reduced body weight, and elevated wheel running when exposed to ABA conditions (Beneke et al., 1995; V. R. Burden et al., 1993; Doerries et al., 1991; Dwyer & Boakes, 1997; Epling & Pierce, 1984; Lett et al., 2001; Routtenberg & Kuznesof, 1967; Tokuyama et al., 1982). Additionally, similar to many other studies, the ABA paradigm increased the weights of the adrenal glands and decreased the weights of the thymus glands (V. R. Burden et al., 1993; Hara et al., 1981; Ness et al., 1995) and spleens (Hara et al., 1981). Moreover, ABA demonstrated reduced body fat when compared to other treatment groups (V. R. Burden et al., 1993). Comparable to other studies, the resistant starch (RS) diet independently decreased retroperitoneal, epididymal, perirenal, and brown adipose tissue (de Deckere, Kloots, & van Amelsvoort, 1995; Francis et al., 2003; Hegsted et al., 2003; Kishida et al., 2001; Pawlak et al., 2001). Lastly, as expected, ABA animals demonstrated elevated levels of catabolic hormones compared to controls (V. R. Burden et al., 1993; Licinio et al., 1996).

Although the ABA outcomes were consistent with many earlier studies, the study did not support our hypothesis that resistant starch (RS) will attenuate the anorexic effects of the ABA model; rather RS exacerbated the conditions of anorexia in these rats. The effects described above were reported at a greater extent in animals fed the RS-diet

than the animals fed the control (C) diet. For example, the ABA animals of the RS-diet group lost an average of 22% and 28% of their pre-experimental body weight, whilst the ABA animals of the C-diet lost an average of 15% and 20% of their initial weight by days 3 and 4, respectively (pre-experimental data not shown). By the end of the experiment (day 5 or 6), the ABA animals fed the RS-diet significantly lost twice as much weight than did the ABA animals fed the C-diet.

In addition, on day 3-4, cumulative data shows that RS-fed ABA animals ran roughly 24% more and ate approximately 19% less g/kg (or 30% less kcal/kg) when compared to ABA animals given the C-diet. Furthermore, sedentary animals on the restricted feeding (RF) schedule in the RS-diet group lost 16% more weight by the end of the study while consuming only 3% less kcal/kg on day 3-4 than did the ABA animals fed the C-diet. A past study by Roy and lab demonstrated that rats fed a low-energy density diet increased food intake to increase energy consumption (H. J. Roy et al., 2003). In this study, ABA animals fed the RS-diet consumed significantly less kcal/kg than did the ABA animals fed the C-diet. Also, the RS-diet, in combination with the ABA paradigm, initiated a significant reduction in brown fat as well as an elevation of norepinephrine levels. These findings strongly show that the resistant starch diet resulted in an accelerated progression to anorexia when used in conjunction with the ABA paradigm.

To our knowledge, studies regarding ABA with respect to diet composition are limited; thus, we attempted to manipulate the diet to examine the possible outcomes. Although RS has been shown to decrease fat pad weights (de Deckere et al., 1995; Francis et al., 2003; Hegsted et al., 2003; Kishida et al., 2001; Pawlak et al., 2001), it has

also been shown to display a gradual rise and steady leveling of post-prandial blood glucose responses rather than producing the transient hyperglycemia and transient hypoglycemia periods as would a high glycemic starch diet (Behall & Hallfrisch, 2002; Hallfrisch & Behall, 2000; Raben, Tagliabue et al., 1994). This demonstration of glucose control may influence the rate of hormone production and secretion, particularly the catabolic hormones (J. E. Hall & Adair, 1998), thereby suggesting that incorporation of resistant starch into the diet can regulate the breakdown and mobilization of energy stores.

In a past study, rats were fed at the onset of the dark cycle and killed at the onset of the dark cycle. The researchers reported significant differences in glucose and insulin responses between ad libitum (AL) and RF treatments (V. R. Burden et al., 1993). Conversely, in our study, the meal period preceded the onset of the dark cycle and the animals were killed at that time as well. There were no significant differences between levels of insulin in ABA animals compared to their respective controls; however, the levels of plasma glucose levels were significantly reduced in ABA animals compared to their controls. It was expected that the glucose levels of the ABA animals on the RS-diet would be higher than those on the C-diet due to the ability of RS to maintain a steady glucose level. However, because the ABA animals from both diet groups were fasted for at least 22.5 hours, it is suggested that the glucose levels of the ABA animals on the RS-diet declined to the levels similar to those on the C-diet. Perhaps, if the samples were taken some hours earlier, there would have been a significant difference between the two ABA groups.

It has been well established that in order to raise blood glucose levels and produce energy for bodily needs, catabolic hormones such as glucagon, norepinephrine, epinephrine, and corticosterone work similarly to promote glycogenolysis and gluconeogenesis, increase lipolysis and ketogenesis, and decrease cellular uptake of glucose (Groff & Gropper, 2000; J. E. Hall & Adair, 1998). We proposed that if the blood glucose levels were maintained at euglycemic levels, the production and release of these catabolic hormones would not be necessary at an elevated rate and the body's energy storage will not be utilized to a great extent.

In our study, the levels of norepinephrine, epinephrine, and corticosterone significantly increased in ABA rats fed the RS-diet when compared to their respective controls. Moreover, only the norepinephrine levels of ABA animals fed the RS-diet were significantly higher than the ABA animals fed the C-diet. On the other hand, only corticosterone levels significantly elevated in ABA rats fed the C-diet compared to their respective controls. Similarly, Burden and co-authors showed an increase in corticosterone levels in experimental animals; however, they found norepinephrine levels to be consistent among all groups (V. R. Burden et al., 1993). Because the ABA animals were exercising and restricted of food for a long period of time, immediate glucose availability was insufficient to meet the demands of the body; therefore, these animals exhibit high secretions of corticosterone. In contrast to our expectations, the glucose levels of ABA animals fed the RS-diet were not maintained at a steady, euglycemic level by the time the meal is usually provided; thus, there were high elevations of all three catabolic hormones (i.e., corticosterone, norepinephrine, and epinephrine).

Because glucagon is a hormone that helps to maintain body homeostasis during times of fasting, plasma glucagon levels were suggested to be elevated in the RF animals (J. E. Hall & Adair, 1998). Contrastingly, in our study, there were no significant differences between ABA groups and their respective controls. On the other hand, Gee and associates found that there was no significant effect of RS on plasma glucagon levels between experimental and control groups (Gee, Faulks, & Johnson, 1991; Gee & Johnson, 1990). Similarly, there were no significant differences between our control groups (i.e., sedentary AL-fed animals) or any other corresponding pairs (i.e., sedentary AL-fed animals, exercising AL-fed animals, sedentary RF animals, or ABA animals) from each diet treatment. This finding suggests that glucagon may not play a role in ABA.

In the fasting state, low levels of circulating insulin and increased levels of norepinephrine and epinephrine promote lipolysis (Groff & Gropper, 2000). Additionally, catecholamines have been suggested to be associated with corticosterone (Gillies & Grossman, 1985; Licinio et al., 1996); particularly, corticosterone is proposed to exert permissive effects on epinephrine and norepinephrine (J. E. Hall & Adair, 1998). Therefore, we suggest that if all three of these levels are elevated, lipolysis will be further stimulated (Devenport, Knehans, Sundstrom, & Thomas, 1989; Guyton, 1987). Evidently, the ABA animals fed RS-diet demonstrated significant elevations of these catabolic hormones, which were at higher levels than the ABA animals of the C-diet, thus, contributing to the greater loss of body weight and body fat. However, ABA animals of the C-diet did not display elevated levels of norepinephrine and epinephrine levels as they did corticosterone levels. Therefore, it is suggested that the body weight

and fat reduction occurred at a lesser extent in the ABA animals fed the C-diet compared to the ABA animals fed the RS-diet because only corticosterone levels were elevated. Literature indicates that epinephrine is more potent than norepinephrine, which functions primarily as a neurotransmitter (Groff & Gropper, 2000); therefore, it cannot be explained why norepinephrine levels in ABA animals fed the RS-diet were higher than those fed the C-diet, while corticosterone and epinephrine levels were not significantly different between the two ABA groups.

Exercise tends to increase norepinephrine concentrations; thus it is believed that exercise-induced food intake suppression has been associated with increased plasma norepinephrine concentrations (Duncan et al., 1985; Guillard, Moreau, Genet, & Klepping, 1988; Kohno et al., 2000). Our study supported the idea that elevated exercise and suppressed food intake both contribute to high norepinephrine levels. The norepinephrine levels in ABA rats fed the RS-diet were significantly higher than their respective controls; however, this did not occur in the C-diet. Furthermore, the ABA of the RS-diet had significantly higher levels of norepinephrine compared to the animals fed the C-diet. Accompanying these elevated levels of norepinephrine were high levels of activity and lower amounts of caloric intake when compared to ABA animals fed the C-diet.

Because the adrenal glands control the secretion of corticosterone, it has been suggested that if corticosterone release is not regulated, high levels of this hormone can significantly impair the immune system, thus, leading to atrophy of the thymus glands (J. E. Hall & Adair, 1998). In our study, ABA animals exhibited lower, atrophic thymus weights as well as significantly higher levels of corticosterone compared to their

corresponding controls. In addition, ABA animals of the RS-diet demonstrated hypertrophic adrenal glands, which further supports the idea that elevated levels of corticosterone from the adrenal glands may hinder immune functions.

Summary and Conclusion

The purpose of this study was to determine whether resistant starch (RS) can ameliorate the anorexia associated with the activity-based anorexia (ABA) paradigm. It was believed that a high GI diet produces transient post-prandial hyper- and hypoglycemia. Conversely, because animals fed RS slowly digest and absorb glucose, they are able to maintain euglycemia for a longer period of time when compared to those fed a high GI diet. Therefore, if euglycemia is sustained, there will be less, if any, stimulation of catabolic hormone release and energy stores will not be catabolized. Higher body stores are suggested to attenuate wheel running associated with ABA (Fish & Lewis, 1996). Therefore, if the animals are fed a diet containing RS while exposed to the standard ABA procedure, the effects of the ABA model will be ameliorated.

Our study showed that this specific procedure developed the typical ABA outcomes in rats. Additionally, the results suggest that the anorexic responses only occurred when restricted-feeding and exercising were administered together. Furthermore, when given RS in the diet, these anorexic responses develop at an even faster rate. Therefore, our diet manipulation suggests a non-beneficial outcome for anorexia. However, these results also indicate a method to accelerate the outcomes of ABA.

By the end of the study (day 5 and 6), ABA rats fed the RS-diet had approximately 30% weight loss compared to the 20% weight loss illustrated by the ABA

rats fed the C-diet. RS-fed ABA rats ran approximately 24% more and ate 30% less kcal/kg compared to the ABA rats fed the C-diet. This finding may explain the reason for the non-significant differences in the adrenal gland weights, brown fat weights, epinephrine levels, and norepinephrine levels between ABA and control rats fed the C-diet. In addition, it was expected that the glucose levels of RS-fed rats be at euglycemic levels; however, meal restriction (22hrs) was probably too severe for the RS-fed ABA rats to maintain the levels that were expected of the low GI characteristic of RS. The faster progression to anorexia and cachexia demonstrated by ABA rats fed the RS-diet can be contributed to low glucose levels, which stimulated corticosterone. Also, food intake suppression and high activity levels played a role in elevating levels of epinephrine and norepinephrine. Collectively, elevated levels corticosterone, epinephrine, and norepinephrine, which have all been implicated in catabolism, contributed to the outcomes seen RS-fed ABA rats.

Therefore, when animals are restricted-fed and allowed access to the activity wheels, the combination of these two stress-factors initiates the anorexic and cachexic responses. This study provided valuable information on the relationship among diet, activity, and food consumption. In our study, RS accelerated the outcomes of ABA; therefore, it served as a means to increase the severity of ABA rather than the opposite. Although RS is not recommended in treatment of activity anorexia, it can be considered in the treatment of obesity or as a weight loss program.

Future Directions

It is important to determine what characteristics are associated with anorexia. More specifically, it would be valuable to determine which factors stimulate, accelerate,

delay, or inhibit the progression to anorexia. Anorexia nervosa is affecting more individuals at a younger age, particularly females between 9-14 years of age, although this is not to exclude those at older ages. Abnormally intense exercising and severe dieting have a dramatic effect on this eating disorder. Therefore, further studies should be conducted to determine what type of diet can provide more efficient immediate energy and stored energy in order to delay the cachexia found in individuals diagnosed with anorexia nervosa. Perhaps a high-energy density diet that provides slow-absorbing glucose can prove to be beneficial. In addition, studies should focus on causes for the initiation of the onset of exercise and sustaining of high levels of activity. Lastly, studies should determine what factors can overcome the exercise-induced food intake suppression. Nevertheless, psychological aspects are not neglected and should also be considered.

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APPENDIX A:
IACUC (LSU)

LSU PROTOCOL FOR ANIMAL CARE AND USE

Instructions for Submission: MUST BE TYPED! (Use additional sheets if necessary and attach to this form or use word processor and add lines). **SUBMIT ORIGINAL plus 12 COPIES** to the IACUC Office (Rm. 1502 School of Veterinary Medicine).

PROTOCOL NUMBER: 01-086

APPROVAL DATE:

SECTION 1: Principal Investigator

Name: Maren Hegsted	Office Phone: 578-1518 Home Phone: 769-3097	E-mail address: mhegsted@lsu.edu
Roy Martin	Office Phone: 578-2284	E-mail address: rjmartin@lsu.edu

SECTION 2: Project Title (Enter the name of your project/course number in the block below)

The effects of low versus high glycemic index (GI) starch on weight gain and fat accumulation.

SECTION 3: Animal Species

Species: Rat		Strain: Wistar Zucker Sprague-Dawley
Number of animals needed: Year 1: <u>130</u> Year 2: <u>170</u> TOTAL: <u>300</u>	Maximum number needed at one time: <u>80</u> Year 1 and 2 Wistar – up to 80 Zucker – up to 120 Sprague-Dawley – up to 100	Number of animals to be placed in each group: <u>6 to 10</u> Year 1 Training: up to 10 rats Study 1: <u>10 rats x 4 groups = 40 rats</u> Study 2: <u>10 rats x 8 groups = 80 rats</u> Year 2 Training: up to 10 rats Study 3: <u>10 rats x 8 groups = 80 rats</u> Study 4: <u>10 rats x 8 groups = 80 rats</u>

Animal housing and veterinary care have been coordinated with DLAM office OR LSU Agricultural Center Unit.

X YES

NO

Name of Animal Housing Representative Contacted (typed): Laurie Henderson

Signature (required):

Location of Animals

	DLAM Vivarium	X	Life Sciences Vivarium
	SVM Barns		SVM Fish Building
	LAES (List Site):		Other (List Site):
	Field Study (Do not complete sections 6, 11, and 12		

SECTION 4: Abstract Plan of Research/Teaching

Provide a brief layman's description of the project in the block below.

There is a possible link between weight gain, fat accumulation, and bone density in a diet comprising or low versus high glycemic index (GI) foods. We will be testing methodology to determine optimum procedures and rat strains to be used in future research projects. The rats will be fed a high GI diet or a low GI diet for 12 weeks. The modifications in GI will be achieved by using a waxy cornstarch (100% amylopectin) for the high GI diet and Hi-maize (40% amylopectin and 60% amylose) for the low GI diet. . Amylopectin is rapidly digested in the stomach and small intestine quickly releasing glucose into the blood stream. Amylose is resistant to amylase hydrolysis and is fermented in the cecum and colon producing short chain fatty acids, primarily acetate, butyrate and propionate. Diets will be based on a modified AIN93-G diet with 35% of calories from fat, 45% from carbohydrate and 20% from protein.

The initial study will consist of 4 diet treatment groups consisting of the two diets (high GI or low GI) and two feeding methods (meal fed or ad libitum). Meal fed animals will be fed twice daily with food available for 30 minutes in the morning and one hour in the evening. There will be a 12/12-hour light/ dark cycle set so the evening meal will be offered at the beginning of the dark cycle. The ad libitum fed rats will have diet available to them 24-hours per day.

Abdominal fat and bone density will be estimated in the beginning and at three-week intervals in the anesthetized animals using a SABRE peripheral dual energy x-ray absorptiometer (pDEXA). Non-fasting blood will be drawn through retro orbital sinus bleeding during the first and last pDexa for leptin analysis. After 12 weeks, rats will be fasted overnight, anesthetized with isoflurane, and blood drawn by cardiac puncture prior to dissection. Abdominal fat depots and

liver will be removed, weighed, and frozen in liquid nitrogen. The rat carcass will be eviscerated and frozen for later proximate analysis. Cecal and colon weight and length will be measured to determine the effects of amylose on colon growth.

The results from this initial study will determine the optimum feeding method (meal fed vs ad libitum) to be used in the subsequent studies.

STUDY 2, 3, 4:

Once the optimum feeding method (meal feed vs ad libitum) has been determined, future studies will determine variances between strain (Wistar, Obese Zucker, Lean Zucker, Sprague-Dawley) and sex (male vs female) using the same diet treatment groups (high GI and low GI). Each study will be comprised of 6 to 10 animals per grouping. All procedures will be duplicated in the same manner as stated above.

SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of Louisiana State University, or that of the LSU Agricultural Center. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the Louisiana State University or the LSU Agricultural Center Animal Welfare Assurance on file with the U.S. Public Health Service.

I further assure the Committee that: **1) I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce significant results are being used in this study.**

	Professor	11/1/2001
Principal Investigator Signature	Title/Rank	Date

Maren Hegsted
(Type Name of Principal Investigator)

	Professor	11/1/2001
Principal Investigator Signature	Title/Rank	Date

R J Martin
(Type Name of Principal Investigator)

	Professor	11/1/2001
Co- Investigator Signature	Title/Rank	Date

Carol O'Neil
(Type Name of Co- Investigator)

Investigator Signature _____ Associate Professor _____ 11/1/2001 _____ Co-
Title/Rank Date

Mike Keenan
(Type Name of Co-Investigator)

Investigator Signature _____ Research Associate _____ 11/1/2001 _____ Co-
Title/Rank Date

Kathleen L McCutcheon
(Type Name of Co-Investigator)

Investigator Signature _____ Research Associate _____ 11/1/2001 _____ Co-
Title/Rank Date

Anne R Francis
(Type Name of Co-Investigator)

(applicable) Signature Title/Rank Date Surgeon (if

(Type Name of Surgeon)

SECTION 6: Special Husbandry Requirements

Do your animals have special needs to be addressed by DLAM?

1. YES

TEMPERATURE RANGE		(F) 70-72		Humidity: _____ (%)	
LIGHT CYCLE (hours light/hours dark)			12/12 0400-1600 light / 1600-0400 dark		
CAGING	Type: _____	Size: _____		Filter tops required? YES (During transport for pDexa)	
G	Stainless Steel wire cages	SINGLE			
BEDDING/LITTER	Type: corncob	Autoclaved? No		Changes/week: _____ We will do	
R					
WATER	Sterile: _____	De-ionized: _____	Acidified: _____	Tap: YES	Other: _____
DIET	Special Feeding Requirements: We will feed				
OTHER SPECIAL NEEDS	Red incandescent light if available				

2. **NO**(If you indicate 'No', your animals will be cared for according to standard operating procedures of DLAM)

3. Not Applicable

SECTION 7: Hazardous Materials

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL ROOM?**

Zoonotic/Recombinant Agents?	Radioisotopes?	Hazardous Chemicals?
YES X NO	YES X NO	YES X NO
Agent(s): EXEMPT	Isotope(s): Are you certified by the Radiation Safety Committee?	Compound(s):
IBRDS Chairman Signature:	YES NO	

Note: If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the Biohazardous Materials Safety Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

SECTION 8: Summary of Procedures

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon. Please include the following information:

4. The rationale for using animals. Why should this study be done? What hypothesis will be tested?
5. How and/or why you selected the animal species indicated.
6. How you arrived at the number of animals to be used.
7. A complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents (**name, dose, volume, route, frequency**) that may be administered. Tables and outlines are helpful to indicate group assignments and study progression.
8. A description of procedures designed to assure that discomfort and injury to animals would be limited to that which is unavoidable in the conduct of scientifically valuable research. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. It is advisable that you obtain input from LSU's Attending Veterinarian (Dr. David Baker) or from another veterinarian familiar with the species to be used.

9. A description of any euthanasia method to be used.

Wistar, Zucker, and Sprague-Dawley Rats will be used as Animal models for evaluating the effects of variable diets of high or low Glycemic Index starches with constant calorie and protein content. Male Wistar Rats will be used in the initial study to determine if there is a significant difference in weight gain, fat accumulation, and bone density between the two starches and between the two feeding methods. Male Wistar Rats between the ages of 7 to 20 weeks show a higher growth curve than most strains, so there is a higher probability of weight gain during the 12-week study. This will allow us to analyze data and make decisions on how to proceed with future studies. Once the methodology is determined, future studies will compare strain and sex. Male and female Wistar, Obese Zucker, Lean Zucker, and Sprague-Dawley rats will be used to determine if those results can be duplicated. By using the selected rat strains as animal models, we are better able to replicate the variation in human populations. Each study will use the same methodology (diet, light cycle, caging, and procedures), with the exception of the feeding method (ad libitum vs. meal fed), since the optimum feeding method will be determined during the initial study.

Rats will be blocked by weight, and randomly assigned to diet treatment groups. Each group will consist of a minimum of 6 to a maximum of 10 rats per group. The initial study will use 10 animals per treatment group (4 groups, N=40). Wistar rats are economical, therefore 10 rats per group will allow for possible loss of animals during anesthesia and retro orbital sinus bleeding. Once we have perfected these procedures, fewer numbers may be needed per treatment group in future studies. The final number of rats used for these studies will be determined after analysis of data from the initial study. An additional ten rats will be ordered from one of the DLAM facilities for training. A DLAM veterinarian will teach staff and students proper methods for anesthesia, retro-orbital bleeding, heart puncture and dissection.

The animals will be single housed in wire stainless steel cages. We will measure food intake, and weigh backs will be used to measure spillage. We will feed all animals and change caging. Clean water bottles and fresh water will need to be provided at all times by the Lab animal staff.

The rats will be fed a high GI diet or a low GI diet for 12 weeks. The modifications in GI will be achieved by using a waxy cornstarch (100% amylopectin) for the high GI diet and Hi-maize (40% amylopectin and 60% amylose) for the low GI diet. Amylopectin is rapidly digested in the stomach and small intestine quickly releasing glucose into the blood stream. Amylose is resistant to amylase hydrolysis and is fermented in the cecum and colon producing short chain fatty acids, primarily acetate, butyrate and propionate. Amylopectin provides 4 kcal/g as glucose while amylose provides about 2 kcal/g as fatty acids in fermentation. Diets will be based on a modified AIN93-G diet with 35% of calories from fat, 45% from carbohydrate and 20% from protein.

STUDY 1:

4 diet treatment groups with 10 Male Wistar rats randomly assigned to each group:

- 1) High GI, meal fed
- 2) Low GI, meal fed
- 3) High GI, ad libitum fed
- 4) Low GI, ad libitum fed

The meal fed animals will be fed twice daily with food available from 0830 to 0900 in the morning and from 1600-1700 in the afternoon. The light dark cycle will be light from 0400-1600 and dark from 1600-0400 so the one-hour evening meal will be offered at the beginning of the dark cycle. The ad libitum fed rats will have diet available to them 24-hours per day with food intake, including

spillage, recorded three times weekly. All Rats will be weighed three times per week. Future studies will use 2 diet treatment groups using the Low GI and High GI diets stated above, and one feeding method.

Isoflurane will be used for anesthesia. Artificial tear ointment will be used in both eyes of each rat while anesthetized to prevent eye damage. Abdominal fat and bone density will be estimated at the beginning and at three-week intervals in the anesthetized animals using a SABRE peripheral dual energy x-ray absorptiometer (pDEXA). During the first and last pDEXA, while still under anesthesia, 400ul of blood will be drawn through retro orbital sinus bleeding for leptin analysis. The pDexa machine is located in the nutrition lab, room 548 of the Life Science building, next to the service elevator directly under the 6th floor Life Science Vivarium. For each of the five scheduled procedures, the rats will be brought, four to six at a time, in shoe boxes with micro isolator lids from their animal room in the Vivarium to the Nutrition lab on the 5th floor. They will be returned immediately after recovery to their original cage. Additional monitoring will take place after the retro orbital bleeding is performed. An antibiotic ophthalmic ointment will be used to treat any animals with visible eye irritation.

After 12 weeks, rats will be fasted overnight and moved individually to the necropsy room in the Life Science Vivarium. They will be anesthetized with isoflurane and up to 10cc of blood drawn by cardiac puncture with a syringe resulting in termination. Cervical dislocation will assure death prior to dissection. Abdominal fat depots and liver will be removed, weighed, and frozen in liquid nitrogen. The rat carcass will be eviscerated and frozen for later proximate analysis. Cecal and colon weight and length will be measured to determine the effects of amylose on colon growth.

SECTION 9: Type of Project

	TYPE A - Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.
X	TYPE B - Pain or distress will be relieved by appropriate therapy.
	TYPE C - Drug intervention for pain or distress would interfere with the protocol. (If this block is checked, specific justification MUST be provided.)

SECTION 10: Check “Yes” or “No” to each of the following questions. On a separate page, provide an explanation for any “Yes” answers that are not included in the above summary.

Provide justification for why the action is needed, and include information in Section 8 above, such as who will perform procedures, how they will be performed, frequency, duration, drugs to be used, dosages, routes of administration, etc. Not all of this information may be needed for every “Yes” answer. The information you provide in this section is very important in highlighting specific points of your study that are important considerations for the IACUC in their review process.

YES	NO	Individual(s) Responsible
-----	----	------------------------------

	X	Will animals be restrained? (<i>Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.</i>)	N/A
X		Will animals be fasted?	OVER NIGHT PRIOR TO SACRIFICE
X		Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Who will administer?.....	HEGSTED, O'NEIL, KEENAN, FRANCIS, MCCUTCHEON
	X	Are neuromuscular blocking agents to be used? Who will administer agents?..... How will animals be monitored?	N/A
	X	Will surgical procedures be employed? Are they: Survival_____ Multiple_____ Terminal Who will perform surgery?..... <u>If survival:</u> 1) Who will be responsible for recovery of the animals?..... .. 2) Who will maintain post-operative records?..... 3) Where will records be maintained? 4) Who will provide post-op analgesics?..... Note: <i>Survival surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.</i>	N/A
	X	Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)?	N/A
	X	Is death an endpoint in your experimental procedure? Note: <i>Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.</i>	N/A
	X	Are there emergency treatments by the DLAM veterinary staff that would not be allowed?	N/A

	X	Are you using wild or exotic species for which permits are necessary? (ATTACH COPY) <i>Note: Permits are required for protocol approval.</i>	N/A
X		Will animals be euthanized during or at the close of the study? Who will perform euthanasia?.....	HEGSTED, O'NEIL, KEENAN, FRANCIS, MCCUTCHEON
	X	Will animals be used for antibody production?	N/A
	X	Will Complete Freund's Adjuvant be used? If yes, please justify based on scientific reasons.	N/A
	X	Will other adjuvants be used?	N/A
X		Will blood be collected? How often? <u>3 TIMES</u> Volume? <u>400ul two times (retro orbital)</u> <u>10ml at euthanasia (cardiac puncture)</u> Who will collect blood?..... <i>Note: Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.</i>	HEGSTED, O'NEIL, KEENAN, FRANCIS, MCCUTCHEON

SECTION 11: Animal Management

Individual (or groups of) animals are identified by (i.e. tag, tattoo): cage cards

Check all applicable below:

CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS	PEST CONTROL
X Call Investigator X Clinician to Treat Euthanasia	X Call Investigator Necropsy Disposal. List any special requirements for disposal?	Call Investigator X Pesticides OK No Pesticides

SECTION 12: Disposition of Animals

What will be done with any animals at the conclusion of the project?

X	Animals will be euthanized.
---	-----------------------------

	DLAM/LAES has permission to REASSIGN animals to another IACUC-approved protocol.
	TRANSFER animals to the following IACUC-approved protocol(s). Please list Protocol Number:
	OTHER (Please state)

SECTION 13: Narrative Statement

Federal regulations mandate that you provide **written, narrative statements** for all projects:

11. that the activities do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.

Note: Address the following items only if you indicated project Type B or C in SECTION 9.

12. that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Indicate what those alternatives were and why they are not appropriate.
13. describing the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

The effects of high and low GI diets on weight gain; fat accumulation and bone density can only be analyzed using live animals. Previous studies using glycemic index starch diets did not allow for maximum weight gain, and did not analyze fat accumulation or variations in bone density. The Web of Science search system was used with the key words: glycemic index, insulin sensitivity, rats, resistant starch, amylose, amylopectin, glucose, weight gain, adipose tissue, and leptin for the years 1984 to present. Search was completed 10/25/2001

The only source of pain for this study will be as a result of the retro orbital bleeding. The animals will be under general anesthesia during the procedure and should have only mild and short-term discomfort after recovery. They will be monitored after the procedure for eye irritation and treated promptly as needed. This method was chosen as the optimum method of blood collection since there is less chance of self-mutilation at the collection site as in other recommended methods, including tail and saphaneous vein bleeding. Web of Science search system was used with the key words: retro orbital bleeding, rats, and blood collection for the years 1984 to present. Search was completed 10/29/2001.

SECTION 14: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below.

RULES AND REGULATIONS TRAINING COURSES					
Name	Rules/Regulations Training Course	Date Attended	Species Wet Lab*	Date Attended	Training and Experience*
Maren Hegsted	X YES NO	3/8/00	YES NO		X YES NO
Carol O'Neil	X YES NO	6/1/99	YES NO		X YES NO
Mike Keenan	X YES NO	3/9/99	YES NO		X YES NO
Kathleen L McCutcheon	X YES NO	11/6/01	YES NO		X YES NO
Anne R Francis	X YES NO	11/6/01	YES NO		X YES NO
Rania Mekary	X YES NO	11/6/01	YES NO		YES X NO

* Exemption based on previous experience with the study species may be obtained by written request to the IACUC.

** Training/Experience in assigned procedures for this protocol.

Who will train individuals for participation in protocol procedures?

Dr. Maren Hegsted

Dr. Rhett Stout

Personnel participating in the project that have not attended the Rules and Regulations Course or the applicable Species Wet Lab, will have **six (6) months** from the approval date of the project to complete them.

Rules and Regulations Courses will be held the first Tuesday of every month from 11:00 a.m. until Noon, in room 1212C, School of Veterinary Medicine. The Wet Labs will be held on the same day beginning at 1:00 p.m. in the DLAM facility. Please call Ms. Dawn Best-Desjardins at 578-9643 to sign up for these courses.

SECTION 15: Occupational Health and Safety

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved directly or indirectly in the study should participate in the Occupational Health Program administered through DLAM and the Student Health Center.

Will project personnel participate in the Occupational Health Program?

13. **YES**

14. ☒ **NO**

If yes, please name participants below, and have them contact Mr. Rick Ramsey at 578-9644 for information.



LOUISIANA STATE UNIVERSITY

AND AGRICULTURAL AND MECHANICAL COLLEGE
Institutional Animal Care and Use Committee
Division of Laboratory Animal Medicine

Animal Welfare Assurance # A3612-01

License # 72-3

Multiple Assurance # M1128

November 14, 2001

Dr. Maren Hegsted
Department of Human Ecology

Dear Dr. Hegsted:

Protocol #01-086, entitled "The Effects of Low versus High Glycemic Index (GI) Starch on Weight Gain and Fat Accumulation" lists you as the Principal Investigator.

I am happy to inform you that your protocol was **approved** by the IACUC during our regularly scheduled meeting held on November 8, 2001. This approval is valid for 3 years and authorizes the use of 300 rats.

In accordance with federal regulations, all personnel conducting animal-based research must receive training in the rules and regulations of animal use, and proper handling methods for the species involved. To meet this requirement all personnel, including yourself, involved with this research project must attend a rules and regulations class. Exemption from participation in the wet-lab, based on previous experience, may be obtained by written request. **Dr. Martin** has six months to satisfy this requirement. **This is the only reminder you will receive concerning this.**

When ordering animals for this project, please provide a copy of this letter to DLAM along with your order. This will help keep better track of the animals being used by various investigators. Thank you!

Sincerely,

Philip Elzer, Ph.D.
Chairman

jdb

APPENDIX B:
AMENDMENT TO IACUC (LSU)



Date: September 3, 2002

To: Philip Elzer Chair,
IACUC

From: Roy Martin, Director
Human Ecology

RE: Protocol #01-086 The effects of low vs. high Glycemic index starch on weight gain and body fat accumulation.

Results from the current protocol suggest that a low Glycemic diet that maintains a more steady control of blood glucose availability, might affect activity based anorexia. Based on this new data we would like to add an additional 190 Sprague-Dawley rats to the protocol for the following three studies.

Activity based anorexia (ABA) is a term applied to an animal model of anorexia. We have used this model in the past (Burden et al 1993), and want to use this model again to develop diets that may reverse the spontaneous anorexia. These studies below will be conducted in sequences that permit us to determine:

- If the model is reproducible and if the animals are capable of recovery from the spontaneous anorexia. It is proposed that stress is a major factor in initiation of anorexia so some measures of stress markers will be made (blood corticosterone, adrenal gland morphology etc.) (study 1, 30 Sprague Dawley rats, 5-6 weeks of age, 6 rats per treatment group)
- If a diet high in resistant starch can reverse the anorexia, and affect gene expression in the brain, and stress markers. (study 2, 80 Sprague Dawley rats, 5-6 weeks of age, 10 or less rats per treatment group)
- If a blocker of corticotrophin releasing factor can enhance food intake in the activity induced anorexia and reverse the weight loss. (study 3, 80 Sprague Dawley rats, 5-6 weeks of age, 10 or less rats per treatment group)

The study groups will contain the following:

Study 1

- Control group fed ad libitum
- Running wheel available 22 hours per day and fed ad libitum
- Meal fed once per day, all they can eat for 90-120 minutes
- Running wheel available for 22 hours and meal fed once per day

Note: This last treatment group is the "activity based anorexia" model. These animal will began to lose body weight and fat, eat less food, and run much more in the running wheel than the other three groups. The study will be terminated when this group has lost approximately 25% of their body weight. The animals will be decapitated for measures of brain neuropeptides, neurotransmitters, and genes expressed in food intake control mechanisms. Thirty rats are requested, with 6 animals per treatment group. 5 extra rats are being requested to eliminate the highest and lowest weight animals from the study. The extra rats will be used to train the graduate student in methodology.

Study 2

Will include the same 4 treatment groups used in study 1 as the control groups plus 4 more groups on the same protocol but with low glycemic index starch added to their diets. 80 rats are requested with 10 animals per group, or less, depending on the results of study 1.

- Control group fed ad libitum \pm low GI starch
- Running wheel available 22 hours per day and fed ad libitum \pm low GI starch
- Meal fed once per day, all they can eat for 90-120 minutes \pm low GI starch
- Running wheel available for 22 hours and meal fed once per day \pm low GI starch

Study 3

Will include similar treatment groups to those used in study 2 except instead of low GI starch the 4 experimental groups will receive a corticotropin releasing factor blocker. 80 rats are requested with 10 or less rats being used per group.

1993 Burden, V. R., B. D. White, R. G. Dean, and R.

J. Martin. Activity of the Hypothalamic-pituitary-adrenal axis is elevated in rats with activity-based anorexia. J. Nutr. 123:1217-1225.



LOUISIANA STATE UNIVERSITY

AND AGRICULTURAL AND MECHANICAL COLLEGE
Institutional Animal Care and Use Committee
Division of Laboratory Animal Medicine

Animal Welfare Assurance # A3612-01

License # 72-3

Multiple Assurance # M1128

September 17, 2002

Dr. Maren Hegsted
Department of Human Ecology

Dear Dr. Hegsted:

Protocol #01-086, entitled "The Effects of Low Versus High Glycemic Index (GI) Starch on Weight Gain and Fat Accumulation" lists you as the Principal Investigator.

I am happy to inform you that your amendment to the above protocol was **approved** at the regularly scheduled meeting of the IACUC on September 12, 2002. This approval authorizes the use of 190 additional rats.

Thank you.

Sincerely,

Philip Elzer, Ph.D.
Chairman

dbd

APPENDIX C:
IACUC (PBRC)

PENNINGTON BIOMEDICAL RESEARCH CENTER
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Dr. David Baker, LSU SVM
PBRC

Dr. Lauri Byerley, PBRC
Dr. Ling Lin, PBRC

Dr. Hans Berthoud, PBRC - Chair

Dr. Randy Mynatt,

Mrs. Diane Olinde, Community Representative
Dr. Matrin Rossmeisl, PBRC
Dr. David A. York, PBRC, Institutional Official
Dr. Aamir Zuberi, PBRC

**GUIDELINES FOR SUBMISSION OF NEW PROTOCOL FORMS AND EXISTING
PROTOCOL AMENDMENTS FOR IACUC REVIEW (12/01)**

NEW PROTOCOL

A new protocol form must be filled out and submitted to the IACUC for approval:

- 1) to receive formal approval to begin experiments/procedures outlined in a **new** protocol;
- 2) if there are changes in major operative procedures in an **existing** protocol (the protocol is not assigned a new number); or
- 3) if there are changes in species in an **existing** protocol (the protocol is not assigned a new number).

PROTOCOL RENEWAL

All existing protocols are evaluated by the IACUC every three years. The term *renewal* refers to IACUC action on a previously approved, but EXPIRING protocol. A new *protocol form must be submitted for a renewal (for a maximum of up to three years). If renewed by the IACUC, the protocol is not assigned a new number when renewed.

PROTOCOL AMENDMENT

A protocol may be amended if the original intent of the protocol does not change. Protocol amendments include:

- 1) requests to extend the time remaining on protocols (not to exceed a total of 3 years);
- 2) changes in dosage;
- 3) changes in animal numbers;
- 4) changes in group designations; or
- 5) changes in time length of experiments.

A new protocol form is not necessary; instead, simply submit a letter to the IACUC, citing the protocol number and title, and outlining the change(s) requested.

INSTRUCTIONS FOR USING ANIMAL CARE AND USE PROTOCOL FORM:

- 1. Changes or revisions may frequently be made by the Institutional Animal Care and Use Committee (IACUC) and this file location will always contain the latest revised version of the form.**
- 2. Retrieve the form. Save the file in another location: In the toolbar, select [File] and select [Save as...] . Give the file any name you prefer.**
- 3. Each section on the form was created as a table; therefore, if extra space is required when typing, the vertical space in the text portion of a section will increase as you type. To begin a new paragraph when typing text, simply hit a hard return [Enter]. If you desire to indent paragraphs in text typing, use a control tab [Ctrl] [Tab].**
- 4. After completing the form, you may want to remove excess hard returns between sections in order to limit the finished protocol form to a minimum number of pages.**
- 5. Should you encounter any problems while using this form, please contact Nancy, David York's office, 3-2577.**

(Revised 12/01/01)

**Pennington Biomedical Research Center
PROTOCOL FOR ANIMAL CARE AND USE**

Submission Instructions: MUST BE TYPED! (Use additional sheets if necessary and attach to this form). **SUBMIT ORIGINAL plus 10 COPIES** to the IACUC Office, Rm. B1022 (Dr. David York's office)

SECTION 1: Investigators

Principal Investigator: Roy J. Martin	Office Phone: 578-2284	Home Phone: 766-2684	Email Address MartinRJ@pbrc.edu
Co-Investigator: David S. Roane	Office Phone: 763-2531	Home Phone: 318 342-1700	Email Address RoaneDS@pbrc.edu

SECTION 2: Project Title (Enter the title of your project in the block below.)

NUTRITION, STRESS AND BODY WEIGHT REGULATION
--

SECTION 3: Investigator's Statement regarding the Assurance for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by Pennington Biomedical Research Center's Policy for the Care and Use of Animals. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the PBRC Animal Welfare Assurance on file with the U.S. Public Health Service.

I further assure the Committee that:

- 1) **I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research.**
- 2) **The investigators and technicians are or will be adequately trained to perform the research techniques required in these studies.**
- 3) **I will use the fewest number of animals required to produce the appropriate statistical power for this study.**
- 4) **The research proposed herein is not unnecessarily duplicative of previously reported research.**
- 5) **For those completing Section 13.2 and 13.3: I have reviewed the pertinent scientific literature and the sources and/or databases as noted in Section 13, and have found no valid alternative to any procedures described herein which may cause more than momentary pain or distress, whether it is relieved or not.**

Roy J. Martin	Professor	10-31-02
Type Name of Principal Investigator	Title/Rank	Date

Principal Investigator Signature

David S. Roane	Adjunct professor	10-31-02
-----------------------	-------------------	----------

Type Name of Co-Investigator

Title/Rank

Date

Co-Principal Investigator Signature

SECTION 4: Animal Species

Species: Rat	Strain: Sprague-Dawley rats and Zucker rats
Sex: <input type="checkbox"/> male <input type="checkbox"/> female <input type="checkbox"/> either <input checked="" type="checkbox"/> both	Weight (or age for rodents): 200~250g(SD rats) 5 weeks (Zucker rats)

Species:	Strain:
Sex: <input type="checkbox"/> male <input type="checkbox"/> female <input type="checkbox"/> either <input type="checkbox"/> both	Weight (or age for rodents):

Species: Rat

Number of animals needed: 950

Year 1: → 470

Year 2: → 240

Year 3: → 240

TOTAL: → 950

Maximum # needed at one time: 100

Animal housing and veterinary care must be coordinated with Comparative Biology.

Signature of Comparative Biology Representative
(required):

--

SECTION 5: Abstract Plan of Research/Teaching (Provide a brief layman's description of the project. This information will help the animal care staff to understand any conditions encountered while caring for your animals. This space will expand as you type.)

The overall goals of the present research plan are as follows:

- 1) identify the neurochemical and physiological mechanisms of stress, hunger, satiety and spontaneous anorexia associated with maintenance of body weight and composition.
- 2) identify nutritional interventions that modify or prevent a) weight gain or regain, b) stress-induced and exercised-induced disruption of homeostasis and behavior in experimental animals.

SECTION 6: Special Husbandry Requirements

Do your animals have special needs to be addressed by Comparative Biology? ☒ Yes
☐ No

If yes, please complete each section that is different from the standard of care for rodents. The standard of care is: plastic shoebox, corn cob bedding, rodent chow, $\approx 21-22^{\circ}\text{C}$ room temperature, $\approx 55\%$ humidity, and tap water.

TEMPERATURE RANGE	21-22o (C)		Humidity: 55 (%)	
LIGHT CYCLE (hours light/hours dark)	12:12			
CAGING	Type: wire hanging	Size: standard	Filter tops required? No	
BEDDING/LITTER	Type:	Autoclaved?	Changes/week:	
WATER	Sterile:	De-ionized:	Acidified:	Tap: Yes Other:
DIET	Special Feeding Requirements: In experiments 9,10, and 11, rats will be tube fed with a commercial semi-purified diet. Dr. Jun Zhou will train graduate student (Bing Li) and student work for this type of special feeding procedure.			
IF USING WIRE BOTTOM CAGES, PLEASE JUSTIFY:	Food intake and spillage need to be measured during the experiment period.			
OTHER SPECIAL NEEDS				

SECTION 7: Hazardous Materials Will zoonotic or recombinant, radioactive, or hazardous chemical agents be PRESENT IN THE ANIMAL ROOM?

Zoonotic/Recombinant Agents	Radioisotopes	Hazardous Chemicals
<input type="checkbox"/> YES <input checked="" type="checkbox"/> NO Agent(s): _____ <input type="checkbox"/> EXEMPT IBRDS Chairman Signature: _____	<input type="checkbox"/> YES <input checked="" type="checkbox"/> NO Are you certified by the Radiation Safety Committee? <input type="checkbox"/> YES <input type="checkbox"/> NO Isotope(s): _____	<input type="checkbox"/> YES <input checked="" type="checkbox"/> NO If yes, you must complete the Hazardous Chemical Form (Appendix A) Compound(s): _____

Note: If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the Biohazardous Materials Safety Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for

animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

SECTION 8: Type of Project (check the appropriate box)

- ☐ **TYPE A** - Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.
- ☒ **TYPE B** - Pain or distress will be relieved by appropriate therapy
- ☐ **TYPE C** - Drug intervention for pain or distress would interfere with the protocol. **(If this block is checked, specific justification MUST be provided in the box below.)**

SECTION 9: Summary of Procedures

Answer each of the following in the box provided. If a section is not applicable, indicate not applicable. Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon. Please include the following information: (each box will expand as needed, as text is added)

- 9.1. **Rationale:** The rationale for using animals. Why should this study be done? What hypothesis/es will be tested?

Experimental Series I will focus on analysis of potential glucose sensing genes and proteins in the CNS. Specifically, these experiments will test the hypotheses that: 1) Glucose sensing gene and proteins are co-localized within the same cell in the brain much like pancreatic islet cells. 2) Intracerebroventricular streptozotocin treatment will reduce the number of cells co-localizing glucose sensing proteins. Experiment 1, 2a, and 2b will address hypotheses 1) and 2) respectively.

Experimental Series II will focus on peptides and neurotransmitters related to ingestive behavior and their interactions with glucose sensing cells in the brain. Specifically these experiments will test the hypotheses that: 1) Streptozotocin will decrease glucoprivic induced hyperphagia, decrease the glucosensing related gene expression, and decrease glucosensing related peptides contents in the brain. 2) Streptozotocin will attenuate the behavioral response to certain ingestive peptides and neurotransmitters that are glucose related. 3) Streptozotocin will decrease the in vitro neurotransmitter release stimulated by glucoprivation. 4) Streptozotocin will decrease the hypoglycemia induced by the hypothalamic administration of glibenclamide. Experiment 3, 4a, 4b, 5, and 6 will test these hypotheses respectively.

Experimental Series III will test the hypotheses that glucose-sensing mechanisms in specialized cells of the brain are altered by obesity, by glycemic state, and by simple states of hunger and satiety. Three robust models of disrupted energy balance and glucose homeostasis will be used. Each model has a different etiology. Fatty Zucker rats, over-/underfeeding rats, and glucose/5-thioglucose intracerebroventricular injected rats will be used in this experiment series. We will test these rats' behavioral responsiveness to glucoprivation/glucose excess and correlate these changes with levels of brain glucosensing genes expression in experiment 7, 8, 9 and 10.

Collectively, these experiments will explicitly test whether specialized glucose sensing cells in the brain play a role in the physiological control of feeding behavior and glucose homeostasis.

9.2. **Species Selection:** How and/or why you selected the animal species indicated?

Most of the basic mechanisms of brain regulation of body weight and energy balance are studied in rats and mice. In addition, it is more economical to use the smaller species of animals at this level of investigation. Once a critical factor is identified for body weight regulation it will be tested in larger species and eventually in human trials after safety concerns are addressed.

9.3. **Experimental Design:** A complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents (**name, dose, volume, route, and frequency**) that may be administered. Tables and outlines are helpful to indicate group assignments and study progression.

Table 1. Number of animals required

Experiment number	Number of animals	Justification
1	50 beta=0.80	Based on power analysis of preliminary data, alpha=0.05,

2a	60 experiments	Empirically based on professional judgment with similar
2b	60 experiments	Empirically based on professional judgment with similar
3	80 experiments	Empirically based on professional judgment with similar
4 a	100 relationships	Adequate number for establishing clear dose/ response
4 b	100 relationships	Adequate number for establishing clear dose/ response
5	40 beta=0.80	Based on power analysis of preliminary data, alpha=0.05,
6	80 experiments	Empirically based on professional judgment with similar
7	40 Zucker rats experiments	Empirically based on professional judgment with similar
8	64 male Zucker rats experiments	Empirically based on professional judgment with similar
9	60 experiments	Empirically based on professional judgment with similar
10	60 experiments	Empirically based on professional judgment with similar
11	60 experiments	Empirically based on professional judgment with similar
12	48 experiments	Empirically based on professional judgment with similar
13	48 experiments	Empirically based on professional judgment with similar
Total rats number		950

Experiment 1. Are cells in the brain expressing the same genes and proteins found in the islets of Langerhans.

This study will use in situ hybridization and immunohistochemical techniques to localize cell populations that co-localize components of the pancreatic glucose-sensing machinery. Total number of 50 Sprague-dawley rats will be used. Half of the rats will be used for in situ hybridization to examine glucosensing gene expression. The other half will be used for immunohistochemistry to test glucosensing protein levels

For in situ hybridization, rats will be killed by decapitation. The whole brain will be collected and snap frozen on dry ice and stored at -80°C until be sectioned on a cryostat. For immunohistochemistry, rats will be euthanized with an overdose of pentobarbital and each rat will be perfused transcardially with heparinized, phosphate buffered saline (~ 150 ml) followed by cold phosphate buffered, 4% paraformaldehyde at 4° C. The brain is removed and postfixed in a 20% sucrose/4% paraformaldehyde solution overnight. The brain is then ready for section on a cryostat.

Experiment 2. Streptozotocin destruction of CNS glucose sensing elements.

One hundred and twenty Spraque-Dawley rats will be anesthetized as described in general anesthesia procedure below, and then placed in a stereotaxic apparatus. Sixty rats will be fitted with third ventricle cannula (experiment 2a) and the rest will be fitted with 4th ventricle cannula (experiment 2b). The detailed methods for insertion of cannula are described under the "General method for brain cannulation" below. Rats will be food deprived for 12 hours prior to injection of streptozotocin (100ug in 5 ul vehicle) or vehicle(5 ul of a freshly made buffer containing 120 mM NaCl and 20 mM NaHCO₃, pH 7.2-7.4). Streptozotocin or vehicle will be injected into the 3rd or 4th ventricle by connecting cannula interal with Gastight syringe though polyethylene tubing. The injection rate will be 1ul/3minutes. The cannulae are then closed and the animal will be placed in a warmed cage to recover. Each animal is allowed to recover for 4 to 10 days after streptozotocin injection. After recovery, each animal will be decapitated or euthanized by an overdose of pentobarbital and perfused (for in situ hybridization and for immunohistochemistry respectively, as described in experiment 1). The pattern of staining is then compared between the vehicle and streptozotocin treated rats.

Experiment 3: Streptozotocin will decrease glucoprivic-induced hyperphagia.

Eighty Spraque-Dawley rats will be treated with streptozotocin or vehicle as described in experiment 2. We will choose either 3rd or 4th ventricle cannulation depending on the results of experiment 2. After a 4 to 10 day recovery period, half of the rats in each group will be evaluated for behavioral response to a glucoprivic challenge (200 mg/kg 2DG, i.p) and the other half will have saline injection as control. Food intakes will be measured at the time point of 30, 60, 120, and 240 minutes after injection. This challenge will allow us to determine that the streptozotocin treatment was effective. Following the behavioral test to a glucoprivic challenge (3-5 day recovery period), rat will be killed to examine ingestive relate peptides and their gene expression by immunohistochemistry or in situ hybridization as described in experiment 1. Distribution of staining for the ingestive peptides or the respective receptors will be compared between the vehicle and streptozotocin treated rats.

Experiment 4:

Two hundreds SD rats will be placed 3rd or 4th ventricle cannulae depending on the results of experiment 2. After 7 to 10 days recovery, the rats will be treated with streptozotocin or vehicle as described in experiment 2. Behavioral tests will be conducted 4~7 days after streptozotocin injection. The behavioral tests will include a glucoprivic challenge, as described in experiment 3, to determine efficacy of the streptozotocin. We will also use 12 h food deprivation to stimulate food intake in these rats. The following peptides and neurotransmitters will be tested on altering deprivation induced feeding in rats treated with streptozotocin. We also will develop dose-response curves for each of the peptides and neurotransmitters according to the table below. The injection volume will be 5ul and the freshly made buffer (120 mM NaCl and 20 mM NaHCO₃, pH 7.2-7.4) will be used to prepare the injection solutions. Due to the difficulty of handling such a

large number of cannulated rats, we will subdivided this experiment in two parts. In the experiment 4a, we will test GLP-1, Amylin, and Leptin. In the experiment 4b, we will test NPY, CRH and GABA . One hundred rats (50 for vehicle and 50 for streptozotocin) will be used in each experiment; each dosage group will have 10 rats. Food intakes will be measured at the time point of 30, 60, 120, and 240 minutes after injection.

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PEPTIDE	EFFECT	DOSES

GLP-1	Suppress food intake	0, 0.5, 1, 5, 10 ug
Amylin	Suppress food intake	0, 1, 10, 25, 100 ug/kg
Leptin	Suppress food intake	0, 1, 10, 25, 100 ug/kg
NPY	Increase food intake	0, 1, 10, 25, 100 ug/kg
CRH	Suppress food intake	0, 1, 10, 25, 100 ug/kg
GABA	Increase food intake	0, 1, 10, 25, 100 ug/kg

Experiment 5:

Forty SD rats will be treated with streptozotocin or vehicle as described above. Seven days after the treatments, brains will be quickly removed by decapitation, and placed in a slicing block. Based on immunocytochemistry and in situ hybridization studies in experiment 1, brain areas that express GLUT-2 and Glucokinase will be dissected, weighed and placed in appropriate chambers of a Brandel Superfusion 2000 apparatus. Tissues will be superfused at 0.1 ml/min with Kreb's Ringer bicarbonate buffer (KRB) with the following composition in mM: NaCl 118.6, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.3, NaHCO₃ 24.6, glucose 33 and pargyline 0.4, a monoamine oxidase inhibitor. KRB is used to collect "basal" release. Basal release will be collected for 90 minutes. Then, KRB containing 10 mM 2-DG will be perfused to stimulate neurotransmitter release ("stimulated" release) for 5 minutes. Stimulated release is carried out to check for viability of the tissue. The flow rate will be 100 ul/minute. The eluate from each chamber is collected at 5-minute intervals with a fraction collector. An internal standard is added to each sample as it is collected. Samples are immediately frozen on dry ice and stored at -70°C until analysis via HPLC.

Experiment 6:

Eighty SD rats will be fitted with bilateral cannula targeted for drug delivery to the dorsal aspect of the ventromedial nucleus of the hypothalamus. Following a seven-day recovery period, half of the animals will be bilaterally injected with of streptozotocin as deccribed in experiment 2, but injection volume will be reduced to 2ul each site. The remaining animals will receive injections of vehicle. Four days later, half of the animals in each treatment group will be injected with 1 nmol(2ul) of glibenclamide through each cannula and the remainder will be injected with solution used for preparation of the glibenclamide (see below). A 30ul blood sample will be collected by tail bleeding (see "General method for tail bleeding") immediately before and every 15 minutes after glibenclamide injection, for a period

of one hour. Blood glucose will be measured by glucometric analysis.

As glibenclamide has limited aqueous solubility, it will initially be dissolved in very small quantities of DMSO. The solution will then be brought up to near final dilutions in a freshly made buffer containing 25 mM NaCO₃, 100 mM NaCl, 4 mM KCl and 1 mM glucose. Final pH will be adjusted to 7.2 with 0.1 N HCl. Control animals will be injected with the drug vehicle (including DMSO, 0.2 to 10%).

The weakest point of this approach is probably that of injecting each animals through the VMH cannula twice. Any amount of injection is likely to produce some cell damage and hence introduce that possibility of artifact. None-the-less, we feel this is a necessary risk given the poor (or unknown) diffusion capacity of streptozotocin through the parenchyma of the brain. If streptozotocin was given by a ventricular route, it might not reach the critical areas in concentration sufficient to produce meaningful biological results in the area in question.

Experiment 7.

We will kill 20 lean and 20 obese Zucker rats (8 weeks old) and collect brains for studies of CNS and pancreatic gene expression of proteins involved in glucose sensing based on results of experiment 1 and 2. Half of the rats in each phenotype will be used for in situ hybridization to examine glucosensing gene expression. The other half will be used for immunohistochemistry to test glucosensing protein levels. The detailed methods for brain tissue collections are the same as in the experiment 1.

Experiment 8.

Sixty-four male Zucker rats, half lean, half obese and aged 6 weeks, will be injected glucose. Since we have validated that 2.5 gm of glucose will suppress food intake in hungry Sprague-Dawley rats and that streptozotocin will attenuate this response, we will conduct a glucose dose response curve (0, 1.0, 1.5, and 2.5 gm glucose in 0.5 ml saline solution, i.p.) in both lean and obese rats. Each dosage group will have 8 rats and each rat will be tested repeatedly at age of 6, 8, 10, and 14 weeks

Experiment 9. Does over- and under- feeding models alter CNS cells of glucose sensing?

Sixty Sprague-Dawley rats, weighing about 250 grams, will be randomly assigned into four groups (n=15 per group). One group will be ad libitum fed as an ad lib intake control group; the other three groups will be tube fed. In tube fed groups, one group (regular fed) will be tube fed 85% of ad lib food intake, the second group (underfed group) will be tube fed 50% of ad lib food intake, and the 3rd group (overfed group) will be tube fed 150% of ad lib food intake. As tube feeding 85% of ad libitum food will cause body weight gain similar to ad lib feeding rats according to our previous study, 85% fed group will be used for normal regular fed control in tube fed rats for comparison. Four parts of a commercial semi-purified diet, which contains 70% polycose, 10% casein, 10% coin oil, 3% cellulose, and 1% AIN vitamin and mineral mix, will be mixed with three parts of warm water to make a liquid diet. The rat will be restrained with the right hand and the feeding tube will be gently introduced through the mouth into the stomach. The freshly prepared meal (5 ml) will be gently delivered from syringe and then the tube will be withdrawn. The amount of food delivered to overfed and underfed rats will be gradually adjusted to the desired level of intake over a period of 3-5 days.

The rats will receive one-third the daily amount of food, three times daily (07:00, 14:30, and 22:30) for 14 days. Body weight will be recorded daily before the 07:00 feeding.

This model has the advantage of having a controlled rapid alteration of adipose tissue mass and the up-regulation of either hunger or satiety signals, thus making it easier to measure changes in gene expression. In addition, since weight gain or weight loss is produced without changing diet composition and in animals of the same genetic background, there are fewer confounding factors to consider. Furthermore, the exact caloric intake, meal size and timing can be controlled, further reducing variability. At the end of the study, rats will be decapitated, brain and pancreatic tissues will be removed for RNA and protein extraction. Real-time PCR and westerns will be used to identify gene expression and proteins that are altered in positive and negative energy states. The gene expression we target will be based on results found in the experiment 1 and 2. Specifically those mRNA and proteins that are reduced or eliminated by streptozotocin treatment.

Experiment 10. Does obesity caused by hyperphagia alter feeding response to glucoprivation by altering CNS cells of glucose sensing?

Sixty Sprague-Dawley rats, weighing about 250 grams, will be placed 3rd ventricle cannula (see General method for brain cannulation" below). After 7 to 10 days recovery, the rats will be randomly assigned into two groups (n=30 per group). One group will be tube fed 85% of ad libitum food and the other group will be tube fed with 150% of ad libitum food. The amount of ad libitum food will be determined according to the results of Experiment 9. The diet composition and the feeding method are the same as those described in Experiment 9. After 14 days tube feeding, rats in each group will be divided into two subgroups: control and 2-DG. Control group will be ICV injected with vehicle (5 ul of a freshly made buffer containing 120 mM NaCl and 20 mM NaHCO₃, pH 7.2-7.4) and 2-DG group will ICV injected with 2-DG (10ug in 5ul of vehicle). Food intake will be recorded at 1h, 2h, and 4h after ICV injection. All rats will be killed four hours after injection, for the measurement of glucosensing related gene expression and protein as described in Experiment 1 and Experiment 9.

Experiment 11. Does energy deficiency caused by underfeeding alter feeding response to glucose by altering CNS cells of glucose sensing?

Sixty Sprague-Dawley rats, weighing about 250 grams, will be placed 3rd ventricle cannula (see "General method for brain cannulation" below). After 7 to 10 days recovery, the rats will be randomly assigned into two groups (n=30 per group). One group will be tube fed 85% of ad libitum food and the other group will be tube fed with 50% of ad libitum food. The amount of ad libitum food will be determined according to the results of Experiment 9. The diet composition and the feeding method are the same as those described in Experiment 9. After 14 days tube feeding, rats in each group will be divided into two subgroups: control and glucose. Control group will be ICV injected with vehicle (5 ul of a freshly made buffer containing 120 mM NaCl and 20 mM NaHCO₃, pH 7.2-7.4) and glucose group will ICV injected with glucose (10ug in 5ul of vehicle). Food intake will be recorded at 1h, 2h, and 4h after ICV injection. All rats will be killed four hours after injection, for the measurement of glucosensing related gene expression and

protein as described in Experiment 1 and Experiment 9.

Experiment 12. Long-term hyperglycemia in brain stem area (including AP/NTS) through fourth ventricle infusion of glucose.

Forty-eight male SD rats will be placed 4th ventricle cannula (see “General method for brain cannulation” below). Following complete recovery from surgery (rats will be considered to have recovered with the return to preoperative food intake level and body weight), an Alzet pump, containing test solution, will replace in the peritoneal cavity and connected with the brain cannula on each rats (see “General method for Alzet pump” below). The rats will be divided into 4 groups according to the following table. This system will deliver our test solution to the 4th ventricle areas at a rate of 0.5 ml /hr for 14 days. Food intakes and body weight will be measured during the experiment period and at the end of experiment, all rats will be killed for measurements of glucosensing related gene and protein as described in experiment 1

Treatments		Number of Animals
Control	Mock CSF fluid	12
Experimental	(plus glucose)	
	5 mM	12
	20 mM	12
	100 mM	12

Experiment 13. Long-term hypoglycemia in brain stem (including AP/NTS) through fourth ventricle infusion of 5-thiogluco-

se. Forty-eight male SD rats will be placed 4th ventricle cannula (see General method for brain cannulation” below). Following complete recovery from surgery (rats will be considered to have recovered with the return to preoperative food intake level and body weight), an Alzet pump, containing test solution, will replace in the peritoneal cavity and connected with the brain cannula on each rats (see detailed method for Alzet pump below). The rats will be divided into 4 groups according to the following table. This system will deliver our test solution to the 4th ventricle areas at a rate of 0.5 ml /hr for 14 days. Food intakes and body weight will be measured during the experiment period and at the end of experiment, all rats will be killed for measurements of glucosensing related gene and protein as described in experiment 1

Treatments		Number of Animals *
Control	Mock CSF fluid	12
Experimental	5-thiogluco-	

5.0 mM	12
20.0 mM	12
100.0 mM	12

- 9.4. **Number of Animals:** How did you arrive at the number of animals to be used. (In other words, is this number required to (reach) obtain sufficient statistical power, or necessary as a continual source of tissue harvest for ongoing work, or a limited number for pilot or feasibility studies, or other.) This may be best accomplished by use of a table.

The number of animals used in each experiment is based on a power analysis. Since the standard deviation of the different measurements determines the number of rats to be used in the above considerations and the standard deviation varies with different measurements, it is necessary that adjustments be made in the estimate of animals needed on each trial as we gain experience with some of these measurements. Usually it requires 8-10 rats per treatment group with 3 to 4 treatment groups per study. For the brain cannulation, 12-15 rats per group will be needed. Studies typically last approximately 6 weeks.

- 9.5. **Non-Surgical Procedures:** Describe each procedure to which any animal in any group may be subjected. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. It is advisable that you obtain input from PBRC's Attending Veterinarian (Dr. David Baker).

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9.6. Surgical Procedures/Ante Mortem Tissue Harvest (complete questions 6 a-i if animals survive surgery/tissue harvest. Only sections a-f should be completed if non-survival surgery/tissue harvest.)

<p>9.6.a) Surgical/harvest procedures (describe in detail; include suture material used):</p>	<p>General method for Alzet pump The Alzet mini-osmotic pump(Alza, Stanford, CA, model 2002: 0.5ul/hr for 14 days) will be filled with the appropriate test solution described in each experiment and placed in 0.9% saline at 37°C overnight. The primed pumps will then be placed subcutaneously in the rats under isofluorane anesthesia. A small incision will be made in the scapulae region of the back, where the pump will be placed. Each incision is closed with suture. The combined uses of mini-osmotic pumps and brain cannulation have been successfully utilized in our lab in the study of obesity. Chronic infusion of test solution into brain will be accomplished by connecting outlet of the mini-pump to an infusion cannula (designed to project 1 mm beyond the guide cannula tip) through silicone tubing (Baxter/Scientific Products) filled with test solution. This system will deliver our test solution to the fourth ventricle at a rate of 0.5 ml /hr for 14 days.</p> <p>General method for brain cannulation: Rats will be anaesthetized with Ketamine/Xylazine/Acepromazine mix (80 mg/Kg Ketamine, 6 mg/Kg Xylazine, 2mg/Kg Acepromazine SQ injection). They will be placed in the stereotaxic apparatus. An incision will be made along the midline and periosteum will be scraped from the skull, while a combination of lidocaine and bupivacaine (1:1 dilution) is infiltrating on the cranium. Site-specific guide cannula (Plastics One Inc., VA) will be placed. Third ventricle 22 G guide cannula will be placed using the following coordinates from the bregma: 2.8 mm posterior, 0 mm lateral, 8.1 mm deep. Fourth ventricle 22 G guide cannula will be placed using the following coordinates from the occipital suture: 2.5 mm posterior, 0 mm lateral, 4.5 mm deep. VMH 22 G guide cannula will be placed at 13 degrees using the following coordinates: 2.8mm behind Bregma and 2.8 mm to the right (or left) of Bregma. The cannula connectors will be fixed to the skull with jewelers screws and cyanoacrylate gel (plastic One). 31G injectors will extend 1 mm below the guide cannula.</p> <p>Placement of ventricular cannula will be verified by measuring angiotensin II response. After a 5-day recovery period, the rats will be injected angiotensin II (150ng in 3ul 0.9% saline) through the cannula and those exhibiting a negative drinking response will be excluded. Placement of site-specific cannula will be confirmed histologically by injected with 0.25 ul India ink and killed 4 minutes later. Brains will be fixed in formalin and the location of the injection sites confirmed histologically. The rats will be allowed to recover from surgery for one week and then baseline food intakes and body weights will be recorded. Food intakes and body weights will be recorded through out the experiment period.</p> <p>General method for tail bleeding: Blood is collected in hand-held, conscious rats by tail bleeding. A small section (2-3mm) is nicking from the end of the tail and 30 to 50 ul blood is collected in an eppendorf tube within less than 2 minutes. A simple gauze will be applied to the end of the tail to prevent further bleeding.</p>
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9.6.b) Personnel responsible for:		Name	Lab Phone	Home Phone	Email address
	1. Surgery/Tissue Harvest	Bing Li Jun Zhou Xiaochun Xi Iwona Bogacka	763-2531 763-2531 763-2531 763-2531	267-6950 819-2286 273-8432 216-0439	Lib@pbrc.edu Zhouj@pbrc.edu xix@pbrc.edu BogackIU@pbrc.edu
	2. Anesthesia	Bing Li Jun Zhou Xiaochun Xi Iwona Bogacka	763-2531 763-2531 763-2531 763-2531	267-6950 819-2286 273-8432 216-0439	Lib@pbrc.edu Zhouj@pbrc.edu xix@pbrc.edu BogackIU@pbrc.edu
	3. Post-op Care	Bing Li Jun Zhou Xiaochun Xi Iwona Bogacka	763-2531 763-2531 763-2531 763-2531	267-6950 819-2286 273-8432 216-0439	Lib@pbrc.edu Zhouj@pbrc.edu xix@pbrc.edu BogackIU@pbrc.edu
9.6.c) Location where surgery/tissue harvest is performed:		PBRC Comparative biology surgery room			
9.6.d) Anesthesia: (Duplicate table if more than one drug. Rodent cocktail would be considered one drug.)	Drug:	#1 - Ketamine/acepromazine/xylazine #2 - 0.25%Bupivacaine/Lidocaine			
	Dose:	#1 - 80/2.0/6.0mg/kg #2 - 2.5~12.5 mg/kg or 1:1 dilution			
	Frequency:	#1 - Once #2 - Once			
	Route of Administration:	#1 - SQ #2 - infiltrating the periosteum at the site where trephining will occur			
9.6.e) Methods of monitoring anesthesia depth (e.g. monitoring heart rate, palpebral reflex):		Monitoring 1) Respiratory rate 2) Mucous membrane color 3) Reflexes			
9.6.f) Paralytic agents and dose (include methods for monitoring anesthetic depth while paralyzed):					

9.6.g) Post-op care (Describe post-op care provided including surveillance.):	1. First 24 hours:	put rats in a warm place and observe rats until they are conscious
	2. Second 24 hours:	observe rat's behavior, body weight, and food intakes
	3. Thereafter:	observe rat's behavior, body weight, and food intakes
9.6.h) Post-op analgesia regimen (Procedures known to cause postoperative pain in humans should be considered painful to the subject animal):	Drug:	Tylenol
	Dose:	110~300 mg/kg body weight (1:1000 dilution)
	Frequency	in drinking water for 4 to 5 days
	Route of Administration:	oral
9.6.i) Are there more than one survival surgery procedures performed on a single animal?	<input checked="checked" type="checkbox"/> YES <input type="checkbox"/> NO	If yes, please provide justification. The combined use of mini-osmotic pumps and brain cannulation can chronically infusion of test solution into certain site of the brain.

9.7. **Euthanasia:** What method or agent will be used to sacrifice the animals? Include dosage and route of administration.

Method:	Animals will be euthanized at the end of the experiment by rapid decapitation. Due to the nature of the biochemical measurements to be performed, this must be carried out without anesthesia. Decapitation will be carried out by individuals who have had previous training and experience with the procedure and can minimize stress to the animal.
Drug:	
Route of Administration:	
Who will perform euthanasia?	Jun Zhou, Iwona Bogacka, Bing Li, Xiaochu Xi and Kichoon Lee

SECTION 10: Procedure Checklist. Check "Yes" or "No" to each of the following questions.

Provide justification for **why** the action is needed. The information you provide in this section is very important in highlighting specific points of your study that are important considerations for the IACUC in their review process.

YES	NO	Question	Response to Question
	No	<p>← .. Will animals be restrained? (Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)</p> <p>If so, how? →</p> <p>Who is the individual responsible? →</p>	
Yes		<p>← .. Will animals be fasted?</p> <p>Is yes, for how long? →</p>	50% feeding for 14 days
Yes		<p>← .. Do you anticipate any adverse effects of the experimental procedures on the animals?</p> <p>If yes, list the conditions (e.g., pain, discomfort, % weight loss, maximum tumor size, fever, minimum packed cell volume, etc)..... →</p>	weight loss
	No	<p>← .. Is death an endpoint in your experimental procedure? Note: <i>Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.</i></p>	Not Applicable
	No	<p>← .. Are there emergency treatments by the Comparative Biology veterinary staff that would not be allowed?</p> <p>If yes, list the treatments. →</p>	
	No	<p>← .. Are you using wild or exotic species for which permits are necessary? (ATTACH COPY)</p> <p>Note: <i>Permits are required for protocol approval.</i></p>	Not Applicable
	No	<p>← ... Will animals be used for antibody production?</p>	Not Applicable
	No	<p>← .. Will Complete Freund's Adjuvant be used?</p> <p>If yes, please justify based on scientific reasons. →</p>	
	No	<p>← .. Will other adjuvants be used?</p> <p>If yes, please specify. →</p>	
Yes		<p>← .. Will blood be collected? If yes: (Note: <i>Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.</i>)</p>	

How often?	Once or twice depending on particular experiment
... →	
Volume?	0.05ml for tail bleeding
... →	
Who will collect blood? →	Jun Zhou, Iwona Bogaka, Xiaochun Xi, Kichoon Lee, and Bing Li

SECTION 11: Animal Management

11.a) Individual (or groups of) animals are identified by (i.e. tag, tattoo):

Cage card

11.b) Check all applicable below:

CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS	PEST CONTROL
<input checked="" type="checkbox"/> Call Investigator <input type="checkbox"/> Clinician to Treat <input type="checkbox"/> Euthanasia	<input type="checkbox"/> Call Investigator <input type="checkbox"/> Necropsy <input checked="" type="checkbox"/> Disposal. List any special requirements for disposal?	<input checked="" type="checkbox"/> Call Investigator <input type="checkbox"/> Pesticides OK <input type="checkbox"/> No Pesticides

SECTION 12: Disposition of Animals (What will be done with any animals at the conclusion of the project? Check the appropriate box.)

- ☒ Animals will be euthanized.
☐ Comparative Biology has permission to **REASSIGN** animals to another IACUC-approved protocol.
☐ **TRANSFER** animals to the following IACUC-approved protocol(s). **Protocol Number:**
☐ OTHER (Please state):

SECTION 13: Narrative Statement

Federal regulations mandate that you provide **written, narrative statements** for all projects:

- 13.1. that the activities do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc)

Source: Electronic database

If your source is an electronic database(s), complete the following boxes.

Date Search Completed: 10-31-02
Database(s) Searched: Medline
Keywords: obesity, food intake, satiety peptides, streptozotocin, glucose signaling, brain,

central nervous system, brain metabolism; attendance at scientific meetings where discussions of regulation of food intake and obesity are held.

Years covered in search: 1966~ present

Note: Answer 13.2 and 13.3 if you indicated Type B or C in Section 8.

13.2. that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Indicate what those alternatives were and why they are not appropriate.

there are no alternatives to the procedures in this proposal

13.3. describing the methods you used to determine that alternatives to such procedures were not available. Put your statements in the block below.

Source: Electronic database

If your source is an electronic database(s), complete the following boxes.

Date Search Completed: 10-31-02

Database Searched: Medline

Keywords: obesity, food intake, satiety peptides, streptozotocin, glucose signaling, brain, central nervous system, brain metabolism; attendance at scientific meetings where discussions of regulation of food intake and obesity are held.

Years covered in search: 1966~present

SECTION 14: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below.

Name	Training and Experience?	If no, who will do training?
David S. Roane	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
Jun Zhou	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
Iwona Bogacka	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
Bing Li/Xiaochun Xi	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	

NOTE: All personnel must complete Comparative Biology's training course in order to have access to Comparative Biology.

APPENDIX A

Hazardous Chemical Form for Chemical Use in Animal Rooms

This form should be completed for all studies in which hazardous agents (e.g., toxic chemicals, or carcinogens) are to be administered to animals that are expected to survive following exposure. A separate form must be submitted for each hazardous agent used. This form must be approved prior to starting a study.

INVESTIGATOR: _____ PHONE: _____ IACUC
PROTOCOL#: _____

Compound to be administered (please attach MSDS):

Site of compound dose preparation and brief description of containment (safety measures including hood, gloves, etc.):

Concentration administered:

Location of compound storage and conditions of storage:

Degree of health hazard to humans (circle the most appropriate number):

LOW 1 2 3 4 5 HIGH

1 = no special precautions required
precautions required

5 = extreme

Assays/Assessment to determine exposure to animal or personnel:

Species to be used: _____ Strain:

Route of administration: _____ Dose:

Duration of administration:

Note the anticipated morbidity (____%) and mortality (____%) if known.

Length of time the animals and/or their environment must be considered hazardous following exposure:

Maximum number of exposed animals that will be maintained at one time:

Indicate the experiment endpoint:

The following personal protective equipment must be used in the room:

- ☐ Lab coat/Coveralls ☐ NIOSH Certified Dust Mask ☐ Fitted Respirator with HEPA filters
☐ Shoe covers/booties ☐ Disposable gloves ☐ Latex
☐ Nitrile gloves ☐ Other:
-

NOTE: Short pants, skirts, and dresses may not be worn in the animal room.

Other relevant information or procedures: include 1) additional precautions, 2) emergency treatments for exposed personnel, 3) decontamination procedures for equipment, 4) housing areas, 5) personnel, 6) how to determine if personnel are or are not exposed:

Is the compound retained in the animal tissue? _____ Yes _____ No (If no, please justify.)

Animal carcasses must be disposed of in the following manner:

Do animals treated with the compound excrete active compounds or metabolites in urine, feces, or other?

_____ Yes _____ No (If no, please justify.)

Contaminated feed, water, bedding, and other waste must be disposed of in the following manner:

Investigator: _____ Date: _____

Chemical Safety Committee approval: _____ Date: _____

IACUC approval: _____ Date: _____

**PENNINGTON BIOMEDICAL RESEARCH CENTER
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE**

Dr. David Baker, SVM
Dr. Lauri Byerley, PBRC
Dr. Thomas Gettys, PBRC

Dr. Hans Berthoud, PBRC, Chair
Mrs. Diane Olinde, Community Representative
Dr. David A. York, PBRC, Institutional Official

Dr. Elaine Hardman, PBRC
Dr. Ling Lin PBRC
Dr. Amir Zuberi, PBRC

November 21, 2002

TO: Dr. Roy Martin, Principal Investigator
Dr. David Roane, Co-Investigator
FROM: Dr. H-R Berthoud, Chairman

HRB

This is to inform you that new protocol #229 "Nutrition, Stress and Body Weight Regulation" was approved by the IACUC at its November 21, 2002 meeting.

The protocol will expire in November, 2005.

Thank you.

APPENDIX D:
AMENDMENT TO IACUC (PBRC)



Pennington Biomedical Research Center
LOUISIANA STATE UNIVERSITY

Date: 1/10/03

To: Dr. David York,
Director of Basic Sciences

From: Roy J. Martin: *Roy Martin*
Army Contract Task 3 leader

Re: Amendment to "Nutrition Stress and Body Weight Regulation" #229, approved Nov. 21, 2002.

Activity based anorexia (ABA) is a term applied to an animal model of anorexia. We have used this model in the past (Burden et al 1993), and want to use this model again to develop diets that may reverse the spontaneous anorexia as part of the Army Contract Task 3. These studies below will be conducted in sequences that permit us to determine if the model is reproducible and if the animals are capable of recovery from the spontaneous anorexia. It is proposed that stress is a major factor in initiation of anorexia so some measures of stress markers will be made (blood corticosterone, epinephrine, adrenal gland morphology etc.) (Study 1). If a diet high in resistant starch can reverse the anorexia and body weight loss, gene expression in the brain, glucose sensing and stress markers will be measured (Study 2).

Study 1 (n=40, 10 per group) The study groups will contain the following

- Control group fed ad libitum
- Running wheel available 22 hours per day and fed ad libitum
- Meal fed once per day, all they can eat for 90-120 minutes
- Running wheel available for 22 hours and meal fed once per day all they can eat for 90-120 minutes

This last treatment group is the "activity based anorexia" model. These animals will begin to lose body weight and fat, eat less food, and run much more in the running wheel than the other groups. The study will be terminated when this group has lost approximately 25% of their body weight. The animals will be decapitated for measures of brain neuropeptides, neurotransmitters, and genes expressed that are related to food intake control mechanisms mentioned in the parent proposal. For the first study 10 rats per group will be utilized. After we gather data on statistical variation for some of the brain measurements we will use information to determine "n" in subsequent studies by power analysis.

Study 2 (80 rats) Diet reversal of stress and anorexia associated with the ABA model. Treatment groups would be the diet with resistant starch (high amylose) vs regular starch (high amylopectin) approximately 25% if the purified diet recommended by ASNS.

To: Dr. David Baker
From: Dr. Roy Martin, Principle Investigator
Dr. David Roane, Co-Investigator

We revised our protocol #229 "Nutrition, stress and body weight regulation" as suggested by the Animal Care and Use Committee. We hope that our revised version will be acceptable.

1. The Principle investigators have read and signed the protocol.
2. The analgesia method has been added in section 9.6a. The same method has already been proposed on the previous version in section 9.6d. Cindy Kloster has confirmed the concentration of drugs.
3. The correct dosages for Ketamine/acepromazine/Xylazine are 80/2.0/6.0 mg/kg, which has been confirmed by Cindy Kloster. These dosages have been proposed uniformly throughout the revised document.
4. The placement of Alzet pumps has been changed to under the skin of the back in section 9.6a.
5. The general method for tail bleeding has been justified according the IACUC recommendations in section 9.6a.

***PENNINGTON BIOMEDICAL RESEARCH CENTER
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE***

Dr. Lauri Byerley, PBRC
Dr. Thomas Gettys, PBRC
Dr. Elaine Hardman, PBRC

Dr. Hans Berthoud, PBRC, Chair
Mrs. Diane Olinde, Community Representative
Dr. David A. York, PBRC, Institutional Official

Dr. Ling Lin PBRC
Dr. Linda Waterman, PBRC Attending Veterinarian
Dr. Aamir Zuberi, PBRC

January 16, 2003

TO: Dr. Roy Martin, Principal Investigator
Dr. David Roane, Co-Investigator

FROM: Dr. H-R Berthoud, Chairman



This is to inform you that the amendment to protocol #229 "Nutrition, Stress and Body Weight Regulation" was approved by the IACUC at its January 16, 2003 meeting.

Thank you.

APPENDIX E:
EXPERIMENT ONE RESULTS

EXPERIMENT ONE RESULTS

As mentioned earlier, the results of experiment one were flawed due to uncontrollable environmental conditions and inefficient supervision of laboratory systems; therefore, the results of experiment one were not discussed in earlier chapters. In this section, the results presented below will represent only that of experiment one.

According to written records by the researcher, days 5-8 of the experiment took place during the most critical time of the category 4 hurricane, Hurricane *Lili*, in October 2002. In the course of Hurricane *Lili*, the university was closed and the public was strongly advised to remain within their homes; however, the animals were continued to be fed and weighed and activity was recorded throughout this time. In spite of the effort to maintain experiment protocol, the expected results of the activity-based anorexia (ABA) model did not occur. The ABA animals did not progressively lose weight (Figure 13), eat less (Figure 14), or run more (Figure 15) as the experiment continued; therefore, the experiment terminated when the animals of the ABA group gained and maintained body weight.

Nevertheless, it is not believed that the experiment protocol failed to produce the ABA results. Rather, it is alleged that the results did not take place due to the malfunctioning of the automated lighting system. Because there was an outage of electricity during these critical days of the hurricane, the lights in the laboratory did not turn off automatically. Therefore, it is believed that because rats are nocturnal animals, the continuous light cycle interfered with the ABA model. The animals did not excessively run on the running wheels; thus, they did not develop a suppression in food

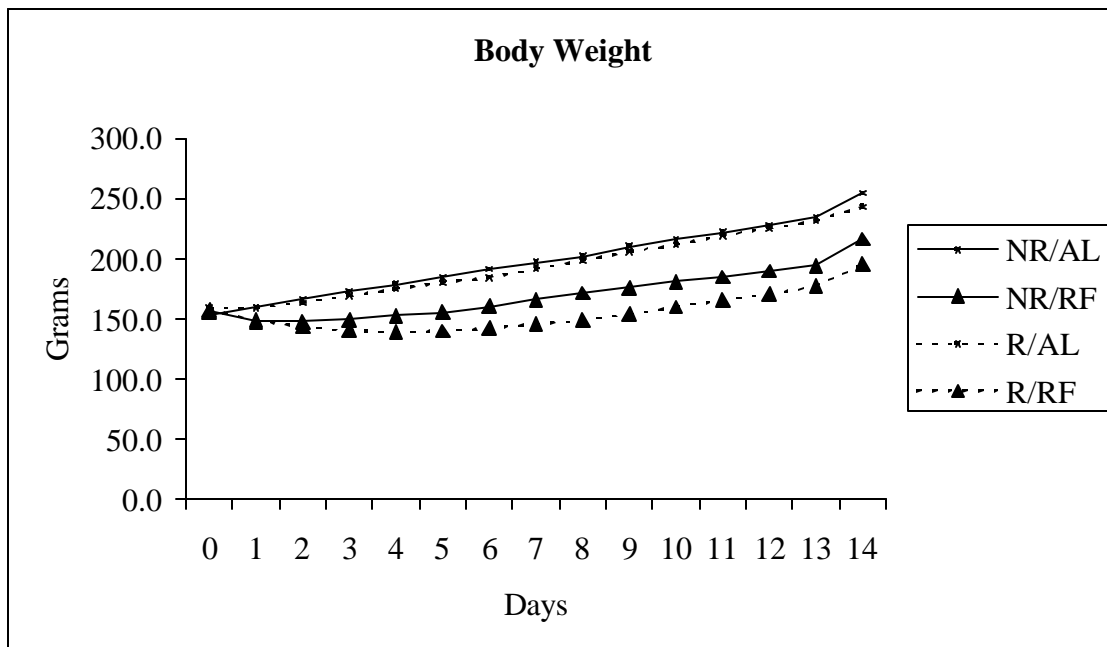


Figure 13. Body Weight (Experiment One)

Note: NR=non-running, R=running, AL=ad libitum, RF=restricted-feeding; day 0 represents the start of the experiment

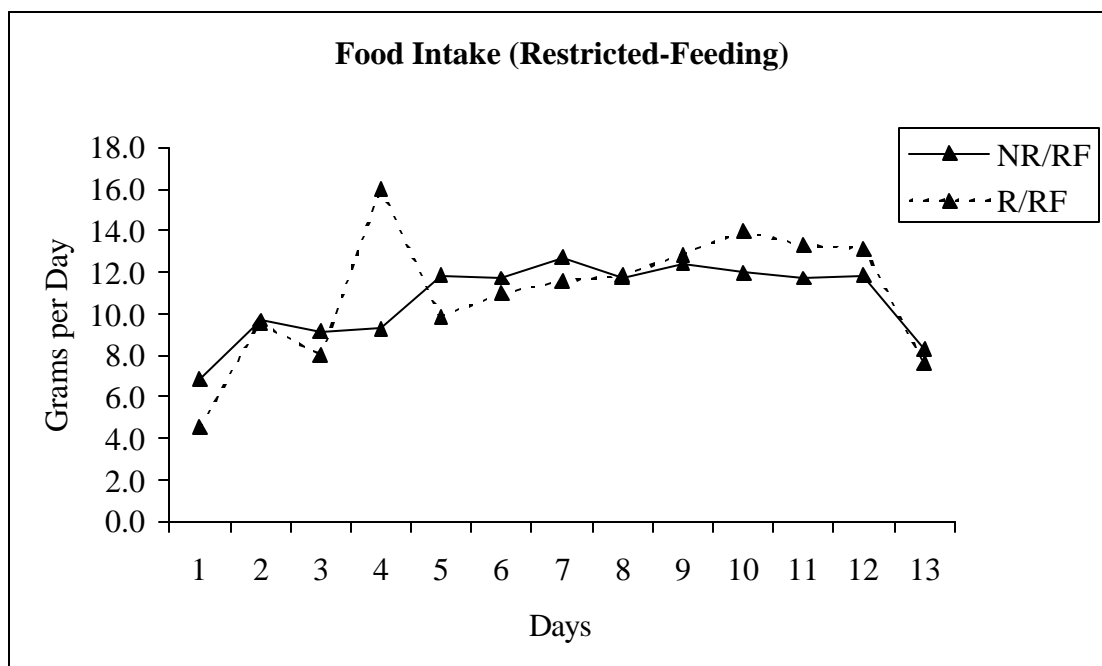


Figure 14. Food Intake (Restricted-Feeding)

Note: NR=non-running, R=running, RF=restricted-feeding; day 1 represents the first one 90-min meal period; values are in grams per day

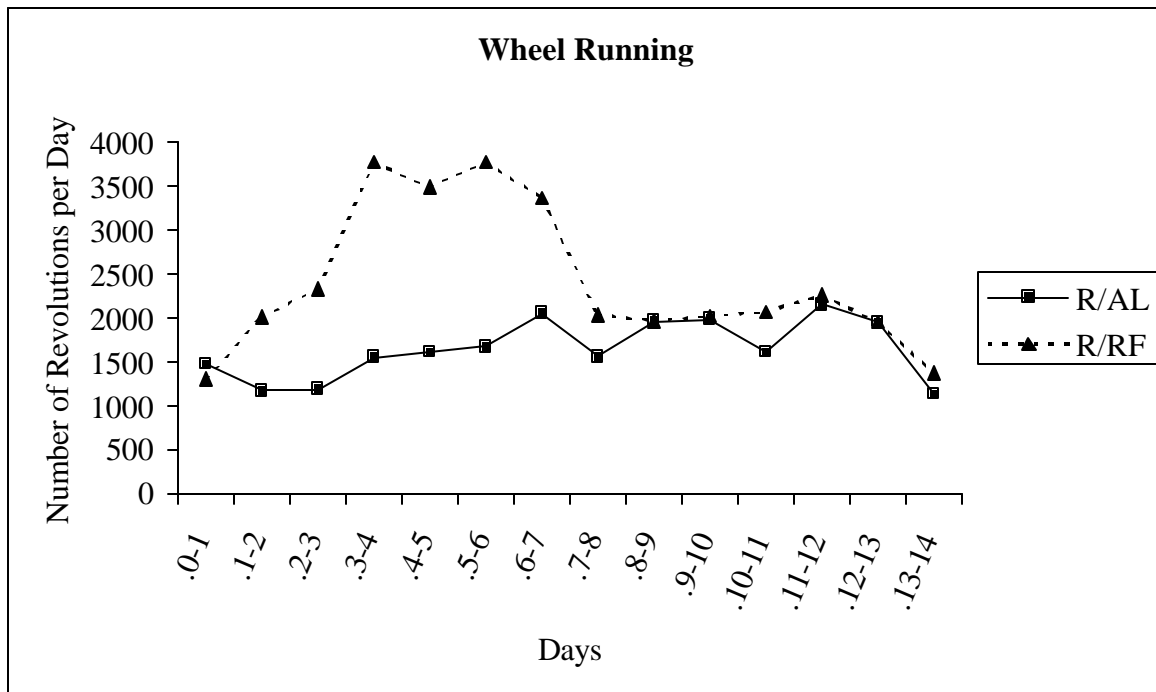


Figure 15. Wheel Running (Experiment One)

Note: R=running, RF=restricted-feeding; day 0-1 represents the first 22hr running period; values are in number of revolutions per day

intake. Because these two essential characteristics of ABA were not demonstrated, the ABA animals survived the ABA model.

The results of this study indicated that the light and dark cycle is very crucial to the ABA model. In addition, it further supports the requirement for elevated activity, and accordingly food intake suppression, to develop activity-anorexia. Without the 12 hour light and dark cycles, the ABA animals will run at a rate comparable to animals exposed to running wheels and fed an ad libitum diet and eat at the amount comparable, if not more, to sedentary restricted-fed animals.

VITA

Holly M. Nguyen was born in Rochester, New York, on December 24, 1980. She graduated from L.W. Higgins High School in Marrero, Louisiana, in May 1998. In May 2002, Holly completed a Bachelor of Science degree in dietetics from Louisiana State University Agricultural and Mechanical (LSU A&M) College. Throughout her undergraduate education, Holly worked as a student researcher assistant in the Political Science Department at LSU A&M. She is currently working as a graduate research assistant at Pennington Biomedical Research Center in Baton Rouge, Louisiana, where she has been during most of her graduate education. Presently, Holly is residing in Baton Rouge, Louisiana, and is pursuing the Master of Science degree from LSU A&M with an emphasis in human nutrition. Holly will receive the degree in May 2004 and continue on to complete the LSU dietetic internship program. She will eventually take the registration exam to become a registered dietitian and pursue her career in clinical dietetics.